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Hypothesis-based Biomarkers for Managing Gastric and Colorectal Cancer Precursors

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M.S.P.H., Peking University, 2009
B.M., Peking University, 2007

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Abstract

Hypothesis-based Biomarkers for Managing Gastric and Colorectal Cancer Precursors

By Huakang Tu

Gastric cancer (GC) and colorectal cancer (CRC) are leading causes of cancer deaths; effectively managing their precursors could reduce the burden from these diseases, but biomarkers are currently not used in their management. The primary objective of this dissertation was to evaluate potential roles for plausible GC- and CRC-related biomarkers for 1) identifying precursor lesions, 2) risk stratification, and 3) surrogate endpoints in chemoprevention trials.

In the first study, I used cross-sectional data from a population-based, serologic/endoscopic screening program for gastric diseases, particularly GC, and found that serum PGII combined with anti-*H. pylori* IgG may be useful for identifying persons with gastric histopathology. In the second study, from the same screening program, I used prospective serological and histological data from repeated blood sample collections and gastric mucosal biopsies from repeated esophagogastroduodenoscopies and found that temporal changes in serum PGI, PGII, PGI/II ratio, and anti-*H. pylori* IgG levels (especially PGII and anti-*H. pylori* IgG combined) are associated with risk for progression of gastric precancerous lesions.

In the third study, I used data from a pilot colonoscopy-based case-control study of colorectal adenoma and found that the mean ratio of rectal TGFα to $TGF\beta_1$ expression and mean rectal TGFα expression in biopsies of normal-appearing colorectal mucosa were higher in adenoma cases than in controls. In the fourth study, I used data from a pilot, randomized, double-blind, placebo-controlled, 2 x 2 factorial clinical trial of calcium 2,000 mg and/or vitamin D_3 800 IU daily over 6 months in sporadic colorectal adenoma patients and found that those in the calcium and vitamin D groups relative to those in the placebo group had non-statistically significant decreases in the mean ratio of rectal TGFα to $TGF\beta_1$ expression in their normal-appearing rectal mucosa.

Taken together, these results suggest that 1) the combination of serum PGII and anti-*H. pylori* IgG could be used to identify, monitor, and manage individuals at increased risk for GC; and 2) the combination of TGFα and $TGF\beta_1$ expression in the normal-appearing colorectal mucosa may be a valid indicator of risk for colorectal neoplasms that may be favorably modifiable by supplemental calcium and/or vitamin D_3.

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Chapter 1. Introduction and Background

Introduction

Gastric cancer and colorectal cancer are among the most common cancers and leading cause of cancer deaths worldwide (1). Gastric cancer, especially the intestinal type, is the end result of progression of precancerous lesions including non-atrophic gastritis, atrophic gastritis, intestinal metaplasia, and dysplasia (2-5). Also, it is widely accepted that most colorectal cancers develop from adenomas over 5-10 years (6-8). This multi-step nature of gastric and colorectal carcinogenesis provides unique opportunities for gastric cancer and colorectal cancer prevention and early detection, which is crucial to reducing the burden of these diseases. It follows that effective management of precursors could lead to reduced gastric cancer and colorectal cancer incidence and mortality and could play an even more important role in reducing the burden than screening for gastric cancer and colorectal cancer themselves, the value of which has already been well recognized.

Currently, there is no consensus on how to manage patients with gastric cancer or colorectal cancer precursors. It has been stated that active surveillance is required for patients with precancerous lesions (9, 10); however, gastric cancer and colorectal cancer develop in “only” a small portion of patients with precursors (11-14); therefore, most persons with these lesions may not need multiple, expensive, invasive surveillance endoscopies—which are not risk free—to prevent the diseases. Of course, the problem is that we currently do not know which individuals fall into these categories, and markers are needed to stratify these patients according to their risk. Currently, management of

gastric cancer and colorectal cancer precursors is only based on histopathological findings, and biomarkers are not yet used to improve management.

Another option to manage patients with precursors of gastric cancer and colorectal cancer is through chemoprevention. However, chemoprevention trials with gastric cancer or colorectal cancer incidence or mortality as the endpoints are limited due to the extended time to develop these cancers and the large sample sizes and high costs involved. Therefore, modifiable pre-neoplastic biomarkers of risk for gastric and colorectal neoplasms that could be used as surrogate endpoints to investigate the potential efficacy of preventive interventions in short-term clinical trials are needed to assess potential efficacy, optimal dose, and safety.

The overall goal of this dissertation is to evaluate potential roles for plausible gastric cancer- and colorectal cancer-related biomarkers for 1) identifying precursor lesions, 2) risk stratification, and 3) surrogate endpoints in chemoprevention trials to assess the potential efficacy, safety, and optimal dose of interventions. The specific research questions are: 1) whether serum levels of pepsinogen I (PGI), PGII, PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG, individually or combined, are sufficiently accurate to be used as biomarkers for identifying abnormal gastric histopathologies, including gastric cancer and its precursors; 2) whether the temporal changes in PGI, PGII, PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG are associated with risk for progression of gastric precancerous lesions; 3) whether the expression of TGFα and/or $TGF\beta_1$ (autocrine/paracrine growth-promoting and -inhibiting factors, respectively) in biopsies of normal-appearing colorectal mucosa differs by colorectal adenoma case-control status (i.e., could be valid biomarkers of risk for colorectal neoplasms); and 4) whether supplemental calcium and vitamin D could modulate the expression of TGFα and $TGF\beta_1$ in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients.

The first two research questions will be addressed by using data from the Zhuanghe Gastric Diseases Screening Program (n = 10,635), a population-based, combined serologic/endoscopic screening program for gastric diseases, particularly gastric cancer, in Zhuanghe County in northern China since 1997 in which repeated gastroscopies with gastric mucosal biopsies and blood sample collections were conducted on 2,039 participants (5,070 person-visits). The third research question will be addressed by using data from a pilot colonoscopy-based case-control study (49 cases / 154 controls), and the fourth research question will be addressed by using data from a pilot, randomized, double-blind, placebo-controlled, 2 x 2 factorial clinical trial (n = 92) of calcium 2,000 mg and/or vitamin D_3 800 IU daily over 6 months. The results may help reduce gastric cancer and colorectal cancer burden by promoting endoscopies (limited resources) in those at highest risk (i.e., most needed) and reducing endoscopies in those at lowest risk (i.e., least needed) and facilitating the process of identifying effective chemoprevention agents.

Background

Morphology of Human Stomach

The stomach is an important organ of the digestive tract and aid in food digestion by secreting protein-digesting enzymes called protease and strong acids. It consists of several regions: the cardia, fundus, body, antrum, and pylorus.

As reviewed by Kouznetsova et al.,(15) there are three histologic zones in the human gastric mucosa: the cardiac, fundus/corpus, and antral/pyloric zones. Surface mucous cells (SMCs) line the gastric epithelium and about 3 million funnel-shaped gastric pits (also called foveolae). At the bottom of these pits is the opening of the gastric glands, which are divided into three regions: isthmus, neck, and base. There are

two gross types of glands present in the different gastric zones: 1) the fundic type, which opens to short pits and are found in the fundus/corpus region, and 2) the antral type, which opens to longer pits and are found in the cardia and antrum/pyloric zones. A pit together with a gland is called a gastric unit. The gastric units in the fundus/corpus zone and the antral/pyloric zone differ in histology, renewal rates, and renewal pattern. In the fundic unit, there are five main cell types: the SMCs,the parietal cells, the mucous neck cells (MNCs), the chief cells, and various endocrine cells. Gastric units undergo bidirectional renewal where daughter cells of the stem and transit amplifying cells located in the isthmus replace the mature cells at the two ends of the units (i.e., the surface epithelium and the bottom of the glands). The stem and transit amplifying cells give rise to the SMCs, the parietal cells, endocrine cells, and MNCs which further generate the chief cells by trans-differentiation as they migrate towards the base of the glands (16). Unlike the fundic unit, the antral unit consists of SMCs, antral gland cells (AGCs), endocrine cells (mainly gastrin producing G cells), and occasional parietal cells, and all cells originate from stem and transit amplifying cells in the isthmus. Also, LGR5+ stem cells located at base of the antrum (probably also the cardia) could migrate up to the isthmus and generate daughter cells (17, 18).

Histopathology of Gastric Cancer

Gastric (stomach) cancer arises from the stomach tissue. Adenocarcinomas, originating from glandular epithelium of the gastric mucosa, account for approximately 90% of all stomach cancers, and Non-Hodgkin's lymphomas and leiomyosarcomas comprise most of the remaining 10% (19, 20). Other rare forms of gastric cancer include adenosquamous, squamous, and undifferentiated carcinomas, choriocarcinomas, carcinoid tumors, rhabdomyosarcomas, hemangiopericytomas, and Kaposi's sarcomas.

On the histological level, there are two main types of adenocarcinomas [the Lauren's classification (21)]: (1) well-differentiated or intestinal type, and (2) undifferentiated or diffuse type. The intestinal type, resembling intestinal carcinomas, forms gland-like structures with cohesive cancer cells and usually occurs in areas of the mucosa with intestinal metaplasia (IM) (22), and this type is related to corpus-dominant gastritis with gastric atrophy and intestinal metaplasia (20). In contrast, for the diffuse type, cancer cells lose cell cohesion, and infiltrate and thicken the stomach wall without forming a discrete and solid mass, and this type is related to pangastritis without atrophy (20).

Also, the two histological types of gastric adenocarcinomas have different epidemiological characteristics (23). The intestinal type is the most common type in high gastric cancer risk areas and the incidence is decreasing. This type is more common in elderly and related to nutritional factors and *Helicobacter pylori* (*H. pylori*) infection. In contrast, the incidence of diffuse type is increasing. This type is more common in young males, and more related to overweight, gastroesophageal reflux, and Barrett's esophagus. However, the causal relationship between *H. pylori* infection and diffuse type is not so strong (23).

Descriptive Epidemiology of Gastric Cancer

New Cases, Deaths: Gastric cancer is the fourth most common cancer worldwide, behind cancers of the lung&bronchus, breast and colo-rectum, and a total of 989,600 incident gastric cancer cases are estimated to have occurred in 2008, accounting for 8% of all the cancer cases (1). However, in developing countries, it is the second most common cancer after lung cancer, and 72% percent of incident cases occur in developing countries (1) and 42% in China alone (24). Gastric cancer is the second leading cause of cancer death worldwide (738,000 deaths, 9.7% of the total),

behind cancers of the lung&bronchus. A total of 556,400 deaths are estimated to have occurred in developing countries, accounting for 75% of deaths worldwide.

Sex, Age: Generally, the incidence rate of gastric cancer in males is twice as high as that in females (1), and the male-to-female ratio ranged from 1.8-1 in Africa to 2.5-1 in the United Kingdom/Ireland (25). The ratio is generally higher in the high risk areas, and in in the older age groups (26); also, higher male-to-female ratios were observed in the intestinal than diffuse gastric cancer (26-28), and in the cardia than noncardia gastric cancer (29).

The incidence rate of gastric cancer increases progressively with age (29). Most cases were diagnosed between the age of 50 and 70 years (19, 20). The cancer rarely occurs before age of 30 years (19), and the diffuse type are relatively more common in younger age groups (21). In the In the United States from 2004-2008, the median age at diagnosis for gastric cancer was 70 years of age; the proportions of cases diagnosed in each age group were: 0.1% under age 20; 1.6% between 20 - 34; 4.7% between 35 - 44; 12.1% between 45 - 54; 18.5% between 55 - 64; 24.2% between 65 - 74; 26.6% between 75 - 84; and 12.2% 85+ years of age (30). In China, the age-specific incidence rates were $4.8/10^5$ under 44, $66.7/10^5$ between 45 and 54, $119.0/10^5$ between 55 and 64, $228.5/10^5$ between 65 and 74, and $261.2/10^5$ 75+ years of age (31).

Geographic Variations: Gastric cancer incidence rates vary by eight to ten-fold across different countries and regions (20, 29), and the age-standardized incidence rates in males ranging from $95.5/10^5$ in Yamagata, Japan, to $7.5/10^5$ in Whites in the United States (19). Japan has the highest incidence rates (age-standardized incidence rates: $69.2/10^5$ in males and $28.6/10^5$ in females) (32). Other high risk areas included Korea, China, Eastern Europe, Central and South America, and moderately high

incidence rates are observed in some countries of Central and West Africa. Southern Asia, North and East Africa, North America, and Australia and New Zealand have the lowest incidence rates (32).

In the United States, the age-adjusted incidence rate was $7.7/10^5$, and the mortality rate was $3.7/10^5$ in both sexes (30); the incidence rates among blacks and Asian/Pacific Islanders were highest, followed by Hispanic American Indian/Alaska Native, and white had the lowest incidence rate (30). In China, the age-adjusted incidence of gastric cancer was estimated to be $37.1/10^5$ in 2005 (31), and mortality rate was $25.2/10^5$ (31).

The incidence rates of noncardia gastric cancer are highly variable, while gastric cancer localized to the cardia has a relatively uniform distribution (19); in terms of histologic type, the differences in incidence rates of intestinal gastric cancer account for most of the international variations, while the incidence of diffuse type is similar across countries (33). Immigrants from high- to low-risk areas had a significantly reduced incidence rate (19). Generally, the first generation of immigrants maintains the risk of the homeland, while the risk of subsequent generation approached that of their new country (34). The reasons for Japan's highest incidence are unclear, and two hypotheses have been proposed. The first one focused on talc, which is a putative dietary carcinogen used to polish rice in Japan (35), and the other focused on the more pathogenic *H. pylori* strains in East Asia (36-38).

Race and Socioeconomics: People in the same geographic region have different risks (19). For example, in the United States, SEER data indicate that the incidences among blacks ($17.2/10^5$) and Asian/Pacific islander ($17.2/10^5$) are about twice of that among whites ($9.5/10^5$), and the incidences among American Indian/Alaska

Natives and Hispanics are $14.7/10^5$ and $14.9/10^5$, respectively (30). While whites have a lower overall gastric cancer risk than blacks, the incidence of cardia gastric cancer is higher among whites compared to blacks (19). In Los Angeles, more detailed incidence data by race were available (29), and in comparison to white population, the age-sex risk ratios were 1.84 for blacks, 1.84 for Chinese, 3.84 for Japanese and Korean. The differences in incidence rates among different races may reflect variance in dietary and environmental factors (27, 39-42), and possibly genetic susceptibility. People with a low socioeconomic status have been shown to have an elevated gastric cancer risk (39). Data from the United States showed the risk of gastric cancer was two times higher in the poor than in the wealthy (27).

Subsite: Generally gastric cancers are categorized based on subsite into gastric cancer subsited in the cardia, as opposed to more distal specified subsites (noncardia). The proportions of carida gastric cancer were 31.7% for males and 18.8% for females, and higher proportions applied to males in each region (25). In 1990, highest proportion was observed in United Kingdom & Ireland, followed by Australia and New Zealand, China, North America, and Northern Europe, and the lowest proportions were observed in Africa, Japan, and Southern Europe (29). In the United States, the proportion of carida gastric cancer was highest among young white males (43).

Time Trend: In the 1930s, gastric cancer was the leading cause of cancer death in the USA and Europe (20, 44). Over the last 70 years, the incidence and mortality rates have declined sharply in many countries around the world, especially in all developed countries (45, 46), and the decline was described as an unplanned triumph (39). Despite of the decline, gastric cancer had been the most common cause of death worldwide until the mid-1990s (46). The most recent worldwide estimates of age adjusted incidence in 2008 (1) ($14.1/10^5$ in both sexes, $19.8/10^5$ in males and $9.1/10^5$ in

females) are about 11% lower than the estimates in 2002 (47) (22.0 /10^5 in males and 10.3/10^5 in females) which are about 11% lower than the estimates in 1998 (48) (24.5/10^5 in males and 11.6/10^5 in females). Paola Bertuccio *et al.* analyzed data from 51 selected registries from 1980 to 2005 and revealed an annual percent decrease of about 3.5% in mortality rate (46).

In the United States, the incidence rate decreased by 1.6% annually from 1975 to 2008 based on SEER data, and the mortality rate decreased by 2.6% annually from 1975 to 1987, 2.2% from 1987 to 1990, and 3% from 1990 to 2008 (30). In China, age-standardized incidence rate decreased from 41.9/10^5 in 2000 to 37.1/10^5 in 2005 among males, and from 19.5/10^5 in 2000 to 17.4/10^5 in 2005 among females (49); the gastric cancer mortality rate decreased from 32.7/10^5 in 2000 to 28.8/10^5 in 2005 among males, and from 15.0/10^5 in 2000 to 13.3/10^5 in 2005 among females. The decline in gastric cancer incidence is possibly due to changes in diet, including a reduced consumption of salted, pickled, and preserved foods, an increased intake of fruit and vegetables, and improvement in food storage using refrigeration (26, 50-53); another possible explanation is the reduction in *H. pylori* infection due to improved hygiene and reduction of crowding which may reduce transmission in childhood (54, 55). However, some data suggest the decline in gastric cancer has applied mostly to intestinal gastric cancer instead of the diffuse type (56, 57).

Despite the overall decline in gastric cancer, cardia gastric cancer rate increased in many regions especially in developed countries in last 30 years (27, 43, 50, 58-60). A report from Olmsted County, Minnesota, U.S. indicated a similar five- or six-fold increase in the incidence of adenocarcinoma of gastric cardia and the lower esophagus in recent decades (61). Another U.S. report using SEER data showed an annual increase of

about 4% from 1976 to 1987 for adenocarcinoma of gastric cardia as well as a parallel increase of adenocarcinoma of esophagus (60).

Survival: In general, five-year survival for gastric cancer patients is poor. Cardia gastric cancer has a much poorer prognosis compared to non-cardia gastric cancer (62), and cardia gastric cancer comprises higher proportions in lower risk countries (29). Japan had the highest five-year survival of 52% possibly due to the mass screening by photofluoroscopy that has been practiced since the 1960s, and the lowest rate was observed in sub-Saharan Africa (6%) (24). The five-year survival was reported to be about 24% in the period 1990–1994 in Europe (63) and other industrialized countries outside of Europe (64, 65). In the United States, the overall five-year survival for 2001-2007 from 17 SEER geographic areas was 26.3% (30). The majority of gastric cancer cases (34%) were diagnosed at a late stage, and the survival among these cases was only 3.6%. However, the five-year survival was much higher (61.4%) among cases diagnosed with localized gastric cancer.

Molecular Basis of Gastric Carcinogenesis

Gastric carcinogenesis is one of the most studied carcinogenic processes and one of the classical examples of a multistage carcinogenesis (2-4, 66). Gastric carcinogenesis is initiated and driven by *H. pylori* induced inflammation with the accumulation of genetic/epigenetic alterations that promote the progression of normal gastric mucosa to chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia/adenoma, and finally to colorectal cancer (67).

Somatic Alterations: So far, no clear linear accumulation of genetic alterations has been recognized in gastric cancer in contrast to the adenoma-colorectal cancer sequence (68) partly due to the fact that the histological subtypes (diffuse vs. intestinal)

of gastric cancer may arise from different pathways (69). Furthermore, intestinal gastric cancer may arise through three different sub-pathways (70).

Genomic Instability: Genomic instability includes a wide range of genetic alterations from point mutations to chromosome rearrangements (71). Two types of genomic instability are generally implicated in gastric cancer (72): chromosomal instability (CIN) and microsatellite instability (MSI), and these two types are not exclusive (73).

CIN: CIN refers to rearrangement and gain or loss of chromosomes resulting in changes in chromosome number (74). CIN is the consequence of failures in either mitotic chromosome transmission or the spindle mitotic checkpoint due to mutations in genes controlling the segregation of chromosome during mitosis (71). As a result of CIN, oncogenes are activated and tumor suppressor genes are inactivated due to high level loss of heterozygosity (LOH), gene deletions and/or amplifications (75, 76). Changes in DNA copy number (high-level amplifications, gains and losses) are common in gastric cancers (68).

Oncogenes that are up-regulated by DNA amplification include *ERBB2, K-SAM and C-MET* (on chromosomal regions 17q12, 10q26, and 7q31, respectively), and tumor suppressor genes that are down-regulated by DNA copy number losses include p16INK, APC, TP53, and deleted in colon cancer (DCC) (on chromosomal regions 9p21, 5q21, 17p13, and 18q21, respectively) (70). In addition, during chromosomal and intrachromosomal instability related gastric carcinogenesis, alterations may occur in bcl-2, SC-1, E-cadherin, β-catenin, K-ras, and vascular endothelial growth factor genes (77). As reviewed by Vauhkonen et al. (68) and Tahara et al (70), and discussed by Choi et al.

(78), in the progression of gastric carcinogenesis, CIN seems to be non-specific and does not follow any consistent pattern.

Frequent LOH has been suggested as an indicator of CIN (78, 79). LOH has been suggested to cause the second inactivating hit of tumor-suppressor gene (the suppressor pathway of carcinogenesis) (79, 80). LOH rate of 26-83% in *TP53* has been reported primarily in intestinal type of gastric cancer (81). LOH at *APC* was found in 22% and 43% in intestinal type and diffuse type, respectively, and the corresponding rates for mutated in colon cancer (*MCC*) gene were 20% and 60% (82). *TFF1* gene was found to be inactivated by LOH in 13-28% of all gastric cancers (83, 84). CDH1 inactivation by LOH is not common in gastric carcinogenesis, ranging from 4.1% to 7.6% (85, 86).

MSI: Microsatellites are repetitive DNA sequences. The length of microsatellites varies from person to person, but for each individual these microsatellites have the same length. However due to replication slippage, mismatch repair impairment or homologous recombination (71), some of these sequences accumulate errors and become longer or shorter. MSI is defined as “a change of any length due to either insertion or deletion of repeating units, in a microsatellite within a tumor when compared to normal tissue (87)”. Replication errors, impairment of base excision repair and mismatch repair, and erro-prone translesion synthesis can lead to mutations including base substitutions, micro-insertions and micro-deletions (71). In particular, functional inactivation by mutations or epigenetic mechanisms in mismatch repair genes MSH2, MLH1, MSH6, PMS1, and PMS2 can lead to MSI (88).

About 15-50% of gastric cancers exhibit MSI (68), with higher percentage in the intestinal type (23-64%) than in the diffuse type (9-24%) (89-94). MSI, leading to

inactivation of other tumor suppressor genes, is likely to play a role in early gastric carcinogenesis, since the presence of MSI was found in areas of intestinal metaplasia or dysplasia in patients with gastric cancer (23).

According to a standard panel of microsatellite markers (Bethesda Guidelines) (87), three levels of MSI can be identified: high-level MSI (MSI-H), low-level MSI (MSI-L) and microsatellite stable (MSS). Generally, MSI-H occurs more frequently in the intestinal type (23-25%) than in the in the diffuse type (4-8%) (78, 93-95). MSI-L was found to be associated with the diffuse type and loss of heterozygosity (LOH) (93, 94). However, the majority (43-92%) of gastric cancers represents the MSS phenotype which was found to be more frequent in the diffuse type (91, 94).

Loss of protein expression of either hMLH1 or hMSH2 was observed in gastric cancers with MSI-H (88), implying that MSI-H is a phenotypic marker for defects in the mismatch repair genes. In particular, silencing of hMLH1 due to promoter hypermethylation was found in most of MSI-H gastric cancers (up to 90%) (96-98). In MSI-H gastric cancers, reading frame shift mutations occurred in several tumor suppressor genes due to alterations in the repetitive sequences of the coding regions. The most frequent affected genes are transforming growth factor b receptor II gene (TGFbR II) (63-92%) and transcription factor gene E2F-4 (37-61%), and other affected genes include insulin-like growth factor II receptor gene (IGFIIR) (5-25%), proapoptotic BAX gene (15-68%), DNA repair genes hMSH6 (22%) and hMSH3 (37-38%) (99-102). Other reported genes that are affected in MSI-H gastric cancers are TCF4, RIZ, CASPASE5, FAS, BCL10, APAF1, MED1, RAD50, BLM, ATR and MRE11 (72). In MSI-L gastric cancers, hMLH1 promoter hypermethylation was found in a significant fraction (up to 50-75%) (97, 98); however, target gene mutations are not frequent (98). The predominant mutations in MSI-L or MSS gastric cancers are in TP53 (88).

***DNA Methylation*:** Hypermethylation of the CpG island in promoter regions, resulting in loss of expression of tumor-suppressor genes, accounts for a majority of epigenetics changes during carcinogenesis (103). As reviewed by Vauhkonen et al., the hypermethylation rate is significantly higher in gastric cancers than in the mucosa of individuals with a healthy stomach (68). In particular, silencing of hMLH1 due to promoter hypermethylation was found in 32% of all gastric cancers (104) and in most of MSI-H gastric cancers (up to 90%) (96-98).

Hypermethylation of the *CDH1* gene is more frequent in diffuse type of gastric cancer (56-83%) than in intestinal type (28-44%) (105-108). Tamura et al. (106) found that the hypermethylation rate of *CDH1* gene among early stage gastric cancer (60%) is similar to the rate among advanced stage gastric cancer (49%), suggesting that hypermethylation of *CDH1* gene is an early event in gastric carcinogenesis. The promoter of the *RUNX3* gene, a novel gastric tumor suppressor gene (109), is hypermethylated in approximately 65% of gastric cancers (110, 111). Using a set of 12 genes, Esterller et al. (104) reported that *APC (34%), p16 (36%), p14 (26%), and MGMT (16%)* are frequently hypermethylated in gastric cancer.

However, in some cases promoter hypermethylation results in activation of genes (69). Human telomerase reverse transcriptase gene (*hTERT*) is one example where promoter hypermethylation is positively correlated with gene expression (112). Reactivation of telomerase activity is necessary for the immortality of cancer cells and is normally inhibited in gastric cells. The activity of hTERT is increased in almost all gastric cancers (70).

Risk Factors for Gastric Cancer

Familial Aggregation: Only about 1-3% percent of all gastric cancers cases are familial cases (68). The familial aggregation of gastric cancer was historically known in the Napoleon Bonaparte family where multiple cases of gastric cancer were noted (113, 114). Generally, among individuals with a positive family history of gastric cancer, a 1.5 to 10.1-fold increased risk was observed (69, 115). However, these results should be interpreted with cautions because most epidemiologic studies used an abbreviated approach to determine family history which may result in misclassification of family history (69). A twin study in Sweden with 23,386 twins revealed a 5-fold increased risk among twins with a partner developing gastric cancer (116); another twin study among Scandinavians with 44,788 twins (117) found that the excess risk was 6.6-fold among dizygotic twin and 10-fold among monozygotic twins and estimated that 28% of the risk was attributed to inherited genes, 10% to shared environmental factors, and the remaining 62% to nonshared environmental factors.

Germline Mutations: A number of syndromes were identified in the families where at least two gastric cancers were found (69): 1) hereditary diffuse gastric cancer (HDGC); 2) familial diffuse gastric cancer (FDGC); 3) familial intestinal gastric cancer (FIGC); 4) familial gastric cancer (FGC, without known histopathology); 5) hereditary nonpolyposis colorectal cancer (HNPCC or Lynch Syndrome, due to mutations in DNA repair genes); 6) Li-Fraumenis syndrome (due to *p53* mutations).

In three Maori families with autosomal dominant diffuse stomach cancer, a germline truncating mutation (G → T nucleotide substitution in the donor splice consensus sequence of exon 7) was first identified in the gene for the cell-cell adhesion protein E-cadherin (*CDH1*) (118), and subsequently this mutation has been detected in families with aggregation of stomach cancer, especially in HDGC (119). E-cadherin, a

transmembrane glycoprotein, plays a crucial role in mediating calcium-dependent adhesion of epithelial cells as well as in maintaining epithelial cells' differentiation and normal tissue morphology (120). A pooled analysis (121) found that among 439 families with any of previously mentioned syndromes, HDGC accounted for 26.9%, FDGC 23.7% for, FIGC 8.7, and FGC 40.8%; while germline mutation in CDH1 was detected in 36.4% of families with HDGC and in 12.5% of families with FDGC. In addition, a germline truncating mutation in *TP53* was identified in a family with both HDGC and Li-Fraumenis syndrome (122). Gastric cancer risk was elevated among patients with HNPCC or Lynch syndrome which is due to inherited mutations that impair DNA mismatch repair, stomach cancer is the third most common cancer and the excessive risk compared to general population was estimated to be from 2.1-fold in Koreans (123) to 4.1-fold among Americans (124). The lifetime risk of gastric cancer in mutated HNPCC gene carriers was estimated to be 19% in the Finish population (125). Germline mutations underlying other syndromes are unclear so far.

Genetic Polymorphisms: The association between genetic polymorphisms and risk of gastric cancer is generally approached by two ways: the candidate gene approach and the genome-wide association study (GWAS) approach. In any approach, the selection of controls and adjustment of confounding are essential in order to get unbiased results. Despite of intensive interest in genetic component of gastric carcinogenesis, we were not able to identify valuable genetic markers for gastric cancer risk.

Candidate Genes Study

Cytokines: Given the inflammatory response to *H. pylori* infection is a driving force for the progression of gastric carcinogenesis, functional polymorphisms in genes

that code for the various cytokines involved in the inflammation have been widely studied. IL-1β plays an important driving role in inflammatory response and is also a potent inhibitor of gastric acid secretion (69). At two of the polymorphisms located at -511 (C-T) and -31 (T-C) in the IL-1β gene, the variant alleles were associated with severe inflammation (69). Also, in IL-1 receptor antagonist gene, a less common allele in intron 2 (*IL-1 RN*2*) is associated with enhanced IL-1β production. These polymorphisms were extensively investigated. In a landmark case-control study, carriers of T allele of *IL-1B-31* and homozygous carriers of *IL-1 RN*2* allele had 1.6- and 2.9-fold increased risk of gastric cancer (126). A most recent meta-analysis including 76 studies showed the and *IL-1 RN* and *IL10-592* polymorphism were associated with modest overall gastric cancer risk, and *IL1B-31* and IL10 -1082 were associated with modest gastric cancer risk among Asian populations. *IL1B-511* polymorphism, *IL1B+3954* polymorphism, *IL8-251* polymorphism (confirmed by another meta-analysis (127)), *IL10-819* polymorphism, *TNFA-308* polymorphism, and *TNFA-238* polymorphism were not associated with overall gastric risk (128).

Cell Proliferation-related Genes: Genes regulate cell proliferation have drawn tremendous attention due to their vital role in carcinogenesis and some of their polymorphisms were widely studied (TP53 Arg72Pro, L-myc EcoRI). A recent meta-analysis including 61 studies summarized the association between gastric cancer risk and polymorphisms of TP53, TP53BP2, Mdm2 (an important negative regulator of the p53 tumor suppressor), p73, Cyclin D1, p16, p21, H-RAS, L-myc, Survivin, DR4, Erf3, KLF6, PPARγ, RUNX3, LAPTM4B, EGF, HER2, TGFB1, TGFBR2, INS, IGF1R, IGF-II, IGFBP1, IGFBP3, IGFBP3, MK, and Pepsinogen (129). In this meta-analysis, only TP53 Arg72Pro was associated with modestly elevated risk of diffuse gastric cancer among

Asians (OR, 1.44; 95% CI, 1.04–1.99), but with decreased risk of intestinal gastric cancer among Caucasians (OR, 0.56; 95% CI, 0.36–0.89).

Metabolism of Carcinogens-related Genes: Human chemical carcinogens are bioactivated or detoxificated by various metabolic enzymes that are potentially important in carcinogenesis. CYP2E1 activates various nitrosamines and other low-molecular-weight carcinogens (130) and its (PstI/RsaI) polymorphism was widely studied with mixed results. A meta-analysis including 13 case–control studies with 2,066 gastric cancer cases and 2,754 controls did not find an overall association of the PstI/RsaI polymorphism with gastric cancer risk; however, when only high-quality studies were considered, an increased risk was associated with the c2 allele (131).

GSTM1 accelerates the binding of glutathione (GSH), a nucleophilic tripeptide, to carcinogens, leading to detoxification of several known chemical compounds. An inherited homozygous deletion of the GSTM1 gene can lead to the absence of GSTM1 expression, hence higher gastric cancer risk of the carriers (132). Ming et al. conducted a meta-analysis including 25 studies, and showed that GSTM1-null genotype was associated with a 1.33-fold increased gastric cancer risk. However, when they stratified on ethnicity, they found that the increased risk was observed in Asians not in Caucasians (132).

DNA Synthesis and Repair-related Genes: Methylenetetrahydrofolate reductase (MTHFR) plays an important role in folate metabolism, deoxynucleotide synthesis, and in DNA methylation, and there are two polymorphisms (C677T and A1298C) that were widely studied in relation to gastric cancer risk. A meta-analysis pooling 16 studies, 2,727 cases and 4,640 controls for C677T and 7 studies, 1,223 cases and 2,015 controls for A1298C showed that MTHFR 677 TT genotype was associated with a 1.5-

fold increased risk of gastric cancer, while no association was found for the A1298C polymorphism (133). Another meta-analysis based on Chinese populations found similar results (134).

X-Ray Repair Cross-Complementing Group 1 gene (XRCC1) protein is a crucial component of the base excision repair pathway which repairs small base DNA damage from oxidation and alkylation (135-137). Three SNPs have been extensively studied: Arg399Gln, Arg194Trp, and Arg280His with gastric cancer risk. A recent meta-analysis including over 12 studies for Arg399Gln, 6 studies for Arg194Trp, and 3 studies for Arg280His showed that these three SNPs were not associated with overall gastric cancer risk (138).

E-cadherin (CDH1): The C-160A SNP of CDH1 in susceptibility to gastric cancer has been studies extensively with mixed results, and three recent meta-analysis, including 14, 17 and 13 studies respectively, did not show a significant association with overall gastric cancer risk; however, when stratified on ethnicity, they found that the direction of association was opposite for Caucasians and Asians (139-141).

*Vascular Endothelial Growth Factor (VEGF)***:** VEGF is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis. The association between its +936 C/T gene polymorphisms and gastric cancer risk was pooled by a recent meta-analysis including 7 case-control studies, which included 1,893 gastric cancer cases and 2,245 controls, and the overall ORs were around 1 and not significant (142).

Genome-wide Association Study (GWAS)

So far, four GWAS studies have been conducted regarding gastric cancer (**Table 1.1**). The first GWAS study was done in Japanese population using a two-stage design, and in this study they found that an intronic SNP (rs2976392) in PSCA (prostate stem

Table 1.1. Summary of the results from GWAS for gastric cancer risk

SNPs	Study 1 (Japanese)	Study 2	Study 3 (Only cardia GC)	Study 4 (Only non-cardia GC)	Overall
rs2976392 (In PSCA)	Yes Diffuse type, not intestinal type GC	No	No	No	Not replicated
rs2274223 rs3765524 rs3781264 rs11187842 rs753724 (All in PLCE1 and high LD)	No	Yes Cardia, not in non-cardia GC	Yes for rs2274223	No	rs2274223 Replicated by two studies
Rs4072037 (In Mucin 1)	No	Yes	No	Yes by imputation	Replicated
rs13042395 (In C20orf54)	No	No	Yes	Yes by imputation	Replicated
Rs13361707 (In PTGER4 and PRKAA1)	No	No	No	Yes	Not replicated
rs9841504 (In ZBTB20)	No	No	No	Yes	Not replicated

cell antigen, possibly involved in regulating gastric epithelial-cell proliferation) was associated with risk of diffuse-type gastric cancer (allele-specific odds ratio (OR) = 1.62, 95% CI = 1.38–1.89, P = $1.11*10^{-9}$), but not with intestinal-type gastric cancer (143).

Another study was conducted in Chinese population with 2,240 gastric cancer cases and 3,302 controls, and multiple variants at 10q23 were associated with gastric cancer risk at a genome-wide significance (144). In particular, rs2274223, a nonsynonymous SNP located in PLCE1, was associated with gastric cancer risk (P = 8.40×10^{-9}; per-allele odds ratio (OR) = 1.31), and the association was only observed in cardia gastric cancer (P = 4.19×10^{-15}; OR = 1.57) but not in noncardia gastric cancer.

A similar GWAS study with 2,766 cases of gastric cardia adenocarcinoma cases and 11,013 controls replicated the association for rs2274223 in PLCE1, and identified another locus on in C20orf54 (rs13042395) (145). These associations are plausible since PLCE1 may play a role in cell growth, differentiation, apoptosis and angiogenesis, while C20orf54 is a riboflavin transporter and deficiency of riboflavin is associated with gastric cancer risk.

The most recent one investigated genome-wide polymorphisms with non-cardia gastric cancer in 1,006 non-cardia gastric cancer cases and 2,273 controls of Chinese descent, and replicated significant associations in an additional 6,897 individuals (3,288 with non-cardia gastric cancer and 3,609 controls) (146). Besides confirmation of previously reported associations of rs2976392 on 8q24, and rs13042395 on 20p13, they identified two new susceptibility loci for non-cardia gastric cancer at 5p13.1 (rs13361707 in the region including PTGER4 and PRKAA1; odds ratio (OR) = 1.41; $P = 7.6 \times 10^{-29}$) and 3q13.31 (rs9841504 in ZBTB20; OR = 0.76; $P = 1.7 \times 10^{-9}$).

Environmental Factors: Non-genetic risk factors associated with gastric cancer have been reviewed extensively elsewhere (19, 20, 52, 147-152) and these risk factors are listed in **Table 1.2**. Carlos A et al. recently summarized the risk factors associated with gastric cancer in terms of strength of evidence (**Table 1.3**) (150). The only two convincing factors are *H. pylori* infection and smoking.

H. pylori: So far, *H. pylori* is the strongest identified risk factor for gastric cancer (153) and has been classified as carcinogenic to humans by IRAC since 1994 (154). Two thirds to three quarters of gastric cancers worldwide are estimated to be associated with *H. pylori* infection (155). *H. pylori* infection is common and it is estimated that half of the world's human population has the infection (156). However only a small portion of

Table 1.2. Risk factors for sporadic gastric cancer (accounts for 97-99% of all gastric cancer cases)
Demographic factors
Older age, male sex, lower socioeconomic status
Infections
H.pylori, Epstein–Barr virus
Dietary, lifestyle, environmental factors
>High salt, high nitrate, High salt, high nitrate, low fruit and vegetable intake, low intake of vitamins A and C, low allium vegetables, low citrus fruit, low green tea, high red and processed meat, high haem iron >Cigarette smoking, alcohol drinking, lower physical activity, >Radiation, asbestos
Medications
low aspirin use , low NSAIDs use, no estrogen treatment,
Family history
Positive family history of gastric cancer
Medical records
Gastric polyps, gastritis, prior surgery for benign conditions, obesity, pernicious anemia,blood type A

Table 1.3. Risk factors for sporadic gastric cancer with level of evidence (Modified from Carlos A et al, (150))		
Evidence	**Decreases risk**	**Increases risk**
Convincing		*H. pylori* Smoking
Probable	Green - yellow vegetables Allium vegetables Fruits and citrus fruits	Salt and salty and smoked foods Alcohol (heavy intake)
Possible	Oestrogen	Red and processed meat Haem iron Obesity (cardia)

infected individuals develop gastric cancer, and evidence showed that *H. pylori* is a necessary but not sufficient cause of non-cardia gastric cancer (157).

Epidemiological Evidence : Nomura et al conducted the first nested case-control study to investigate the association between *H. pylori* infection and gastric cancer among a cohort of 5,908 Japanese American men living in Hawaii over 20 years (158). By the end of the follow-up, 109 cases of pathologically confirmed gastric cancer have been identified, and 109 matched controls were selected. The stored serum of each case and control was tested for seropositivity of IgG antibody to *H. pylori*. Ninety-four percent of gastric cancer cases and 76% of controls were seropositive, resulting in an OR of 6.0 (95% CI: 2.1-17.3). When they limited their analysis to the gastric cancer cases who were diagnosed 10 or more years after the collection of serum sample, the association was stronger (OR=10.5, 95% CI: 2.5-44.8). In another nested case-control study conducted in the USA (159), *H. pylori* infection, as determined by seropositivity of IgG antibody to *H. pylori,* was associated with an OR of 3.6 (95% CI: 1.8-7.3). In a third nested case-control study conducted in England with a similar design (160), an OR of 2.77 (95% CI: 1.04-7.97) was reported.

In a prospective cohort study conducted by Nomura et al. (153), 1,526 Japanese with duodenal ulcers, gastric ulcers, gastric hyperplasia, or nonulcer dyspepsia at the time of enrollment were followed for about 7.8 years, and *H. pylori* infection was assessed by histologic examination, serologic testing, and rapid urease tests and was positive if any of the three tests was positive. Among 1,246 *H. pylori* infected patients, 36 developed gastric cancers, while none developed gastric cancer among 280 patients without *H. pylori* infection.

In 1998, Huang et al. conducted a meta-analysis of the relationship between *H. pylori* seropositivity and gastric cancer (161), and they included 5 nested case-control studies, and 14 case-control studies with 2491 patients and 3959 controls. The summary ORs were 1.92 (95% CI: 1.32–2.78), 2.24 (95% CI: 1.15–4.4), and 1.81 (95% CI: 1.16–2.84) for all studies, nested case-control studies, and case-control studies, respectively. Stronger associations were found when only early stage (OR=6.35) or noncardia (OR=3.08) gastric cancers were included.

In 2001, the *Helicobacter* and Cancer Collaborative Group conducted another meta-analysis (162) including 12 case control studies nested within prospective cohorts with 1,228 gastric cancer cases and 3,406 controls . Studies were eligible only if serum samples were collected before diagnosis of gastric cancer in cases. Overall, the OR for the association between *H. pylori* infection and gastric cancer development was 2.36 (95% CI=1.98–2.81). However, they found that the association was restricted to noncardia cancers (OR=3.0; 95% CI 2.3–3.8), while no association was found in cardia cancers (OR=1.0, 95% CI=0.7–1.4). The association with noncardia cancers was stronger when blood samples for *H. pylori* serology were collected 10 or more years before cancer diagnosis (OR=5.9, 95% CI=3.4–10.3).

According to a relatively recent meta-analysis (163), *H. pylori* eradication treatment reduces the risk for gastric cancer. This meta-analysis included six randomized trials, mostly conducted in Asia. Overall, 37 of 3,388 (1.1%) treated patients developed gastric cancer compared with 56 of 3,307 (1.7%) untreated (control) participants, resulting in a risk ratio (RR) of 0.65 (95% CI=0.43-0.98).

Natural History of H. pylori Infection: *H. pylori,* first isolated by Warren and Marshall in 1983 (164), is a gram-negative spiral-shaped bacterium that colonizes on the

luminal surface of the gastric epithelium, occasionally in an intracellular location (165). *H. pylori* possesses several mechanisms to survive and persist in human gastric epithelium; for example, its highly active urease hydrolyzes urea to ammonia and carbon dioxide which buffer the acidic environment in stomach (166), and its helical morphology and unipolar flagella enable the bacterium move within the mucous layer overlaying gastric epithelium cells (167).

H. pylori infection is generally acquired during childhood by oral ingestion of the bacterium within families (156, 168) and everyone infected with *H. pylori* develops coexisting gastritis, which usually persists for decades unless treated (169). However, spontaneous elimination of *H. pylori* infection occurs, especially in young children and the elderly (170, 171). Spontaneous elimination of the infection occur when the gastric mucosa has become hostile for *H. pylori* colonization, as may happen during extensive intestinal metaplasia of gastric epithelium to which *H. pylori* does not adhere well (172). The outcomes of chronic *H. pylori* infection are diverse and include asymptomatic *H. pylori* infection, duodenal ulcer, MALT lymphoma, gastric ulcer, and gastric cancer (168).

The usual site of infection is the antrum of human stomach (173) where the pH value is higher than the acid secreting fundus. However, under PPI treatment, the bacterium is found in the fundus rather than the antrum (174). In some patients (1%), the infection is predominantly at the fundus which may be due to the specific *H. pylori* strain or a different pH level at the surface of the fundus in these patients (166, 174). Fundic infection is necessary for gastric intestinal metaplasia and this may lead to increased risk of gastric cancer (166, 174).

H. pylori Constituents that Mediate Oncogenesis: Multiple *H. pylori* virulence factors may mediate oncogenesis, and these factors mainly include outer membrane

proteins (OMP), Vacuolating Cytotoxin (VacA), and Cytotoxin-Asscociated Antigen A (cagA).

Outer membrane proteins (OMP): Most of *H. pylori* reside within the mucous gel layer of stomach on top of the apical surface of the gastric epithelium, but approximately 20% bind to gastric epithelial cells (169). *H. pylori* expresses multiple OMPs, and several of these OMPs bind to receptors on gastric epithelial cells.

BabA, one of the OMPs encoded by *H. pylori babA2* gene, is an adhesin that binds to the Lewis histo-blood-group antigen Le^b on gastric epithelial cells (175-177). *H.pylori babA2* positive strains were associated with a higher gastric cancer risk (175). SabA is another *H.pylori* adhesin that binds the sialyl-Lewisx (Le^x) antigen on gastric epithelial cells, (178, 179) and SabA is associated with higher *H. pylori* densitiy(180) and higher gastric cancer risk.(181) *H.pylori* contains various human Lewis antigens, including Le^x, Le^y, Le^a and Le^b, and *H.pylori* may eascape host immune defenses by inhibiting formation of antibodies against shared epitopes in both *H. pylori* and human gastric epithelial cells. OipA, another OMP, may co-regulate the expression of proinflammatory cytokines, including IL-8, IL-6 (182-184) and activate β-catenin (185, 186).

Vacuolating Cytotoxin (VacA): VacA, endcoded by the *H. pylori vac A* gene, is a secreted protein that induces vacuolation in cultured epithelial cells (169). Unlike the *CagA gene*, the *vacA* gene is present in all strains, but it is variable in the 5' signal terminus region (allele types, s1a, s1b, s1c, and s2), the mid-region (m1 and m2), and the intermediate region (i1 and i2) (187). Secreted VacA undergoes proteolysis to yield two functional fragments, p33 and p55, which are involved in pore formation and cell-binding, respectively (169). Full-length VacA binds to multiple epithelial cell-surface

components, including the transmembrane protein, receptor-type tyrosine protein phosphatase-ζ (PTPRZ1) (188), fibronectin (189), epidermal growth factor receptor (EGFR) (190), various lipids (191), and sphingomyelin (192),as well as CD18 (integrin β2) on T cells (193). The toxin inserts itself into the cell membrane and forms a hexameric anion-selective, voltage dependent channel through which bicarbonate and organic anions can be released (194). VacA induces vacuoles of late endosomal origin (169).

VacA not only induces vacuolation but also stimulates apoptosis in gastric epithelial cells (195). Full-length VacA or p33 induces the release of cytochrome c from mitochondria, resulting in activation of caspase 3. VacA containing s1 signal allele induces higher levels of apoptosis than VacA containing s2 signal allele (196). Also, VacA affects host immune response. VacA that binds to integrin β2 on T cells inhibits antigen-dependent proliferation of T cells by interfering interleukin-2 (IL-2) signaling (197).

Clinical studies suggested that vacA s1 and m1 strains that are more virulent than vacA s2 and m2 strains are associated with higher levels of inflammation, epithelial damage, and a higher risk of gastric cancer (150). Patients with gastric cancers usually have the s1 and m1-type *vacA* (175, 187, 198-202). In region with high gastric cancer risk, such as Japan and Columbia, s1/m1-type vacA is the dominant type which may contribute to the high incidence of gastric cancer (203). One recent study showed that i1 allele in the intermediate region was associated with higher risk of gastric cancer (187).

Cytotoxin-Asscociated Antigen A (cagA): The *cagA* gene is located at one end of the cag pathogenicity island (PAI) (204) which contains 31 putative genes including *cagA* gene and those encoding components of a bacterial type IV secretion system (37).

H. pylori species are divided into *cagA* positive and *cagA* negative according to the presence of *cagA gene*. The *cagA* positive strains are more virulent and induce higher grades of gastric inflammation (205). Transgenic expression of CagA in mice resulted in increased gastric epithelial cells proliferation and formation of carcinoma (206), while decreased apoptosis (207), suggesting this molecule as a bacterial oncoprotein.

CagA is translocated into host cells by the type IV secretion system that is encoded by genes located in the *cag* PAI of *H.pylori (208)*. Following the injection, CagA concentrates at the plasma membrane and undergoes tyrosine phosphorylation by multiple members of the SRC family of kinase including SRC, FYN, lYN and YES (209, 210).

CagA tyrosine phosphorylation occurs at EPIYA motifs (211, 212) in four distinct EPIYA sites-EPIYA-A,-B,-C and –D (37). The Western (e.g. Europe, North Amercia and Australia) CagA possess EPIYA-A, EPIYA –B, and 1-3 repeats of EPIYA-C site which is the most commonly phosphorylated site (37), and the most common Western CagA has only a single EPIYA-C (213). In contrast, East-Asian (e.g. Japan, Korea, and China) CagA possess EPIYA-A, EPIYA –B, and EPIYA –D site which is the most commonly phosphorylated site (37, 212). Biological activity of CagA is determined by variation in the EPIYA sites; CagA having more EPIYA-C repeats is more active and East-Asian CagA is more active than Western CagA (212).

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the EPIYA sites; CagA having more EPIYA-C repeats is more active and East-Asian CagA is more active than Western CagA (212).

Phosphorylated CagA specifically binds to and activates a cytoplasimic phosphatase called SHP2 (37). Activated SHP2 can upregulate extracellular signal-regulated kinase (ERK) signaling by RAS- dependent and RAS–independent mechanisms. Sustained ERK activation leads to abnormal mitogenic signal, cell spreading and elongation (214) ("humming-bird phenotype"), cell scattering (214) and apoptosis (37). However, non–phosphorylated CagA also plays a role in carcinogenesis. Non–phosphorylated CagA interact with E-cadherin, the hepatocyte growth factor receptor MET, the phospholipase Cγ (PlCγ), the adaptor protein growth factor receptor-bound protein 2 (GRb2) and the kinase PAR1B (also known as MARK2), which leads to pro-inflammatory and mitogenic responses, the disruption of cell–cell junctions and the loss of cell polarity (215-217). In addition, CagA activate β-catenin pathway by liberating β-catenin from β-catenin-E-cadherin complexes at the cell membrane, allowing β-catenin accumulation in cytoplasm and nucleus (217). Also, CagA, potentially by binding to MET, activates PI3K, leading to inactivation of GSK3 β and subsequent release of β-catenin from the APC- GSK3 β-Axin-β-catenin complex (218).

In gastric epithelial cells, most of phosphorylated CagA binds to SHP2, while a small portion binds to CSK (219), a kinase that negatively regulates SRC-family kinases and subsequently reduces the amount of phosphorylated CagA and actived SPH2.

Another consequence of *cag* PAI is the delivery of peptidoglycan into host cells (169). Peptidoglycan binds NOD1 (220) which activates NF-κb, p38, ERK and IRF7 to induce pro-inflammatory cytokines (220, 221). In addition, peptidoglycan activates PI3K,

leading to inactivation of GSK3 β and subsequent release of β-catenin from the APC-GSK3 β-Axin-β-catenin complex (222).

Epidemiological studies have shown that cagA seropositivity was associated with an increased gastric cancer. According to a meta-analysis including 16 qualified case-control studies with 2284 cases and age- and sex-matched 2770 controls (223), compared with *H.pylori* negative individuals, those who were cagA seropositive had a 2.87-fold increased risk of gastric cancer. Among *H.pylori* infected individuals, cagA seropositivity was associated with an increased risk of 1.64 (95% CI, 1.21-2.24) for gastric cancer overall and of 2.01 (95% CI, 1.21-3.32) for noncardiac gastric cancer. Cardia gastric cancer was not associated with cagA seropositivity.

Human Immune Response to H. pylori: *H. pylori* colonization can induce strong and persistent humoral and cellular immune response at the local and systemic level including an innate and an adaptive response along with varying degrees of epithelial cell degeneration and injury (224, 225).

The innate response is an initial non-specific process, and bacterial contact with monocytes and other APCs induces secretion of proinflammatory cytokines, which recruit granulocytes with the purpose of killing the organism (224).

In contrast, the adaptive immune response is a delayed, antigen-specific process with the activation of T-, B- and memory cells (224). After the activation of T cells, CD4 T-cells differentiate into Th1 cells, secreting IL-2 and interferon- γ and Th2 cells, secreting IL-4, IL-5, and IL-10 (224, 226). Th2 cells simulate B cells in response to extracellular pathogens, while Th1 cells response to intracellular pathogens (168). Because most *H.pylori* resides as an extracellular pathogen, we would expect Th2-cell response; however, H.pylori-specific gastric mucosal T cells are predominately Th1 cells

(227, 228). Th1-cell response leads to cell-mediated immunity and the production of opsonizing antibody classes (predominantly IgG), while Th2-cell response leads to the production of IgM, IgA, and IgE which provides humoral immunity (224). Th1-cell response is associated with increased severity of gastritis but lower *H.pylori* density (226).

Mechanisms of H.pylori-induced Gastric Carcinogenesis: *H. pylori*-induced gastric carcinogenesis was summarized in **Figure 1.1**. The Reactive oxygen and nitrogen species produced in gastric mucosa during *H.pylori*-induced inflammation cause oxidative stress and apoptosis in gastric epithelial cells (229). Studies showed that *H.pylori*-induced inflammation could induce DNA damage in mice (230, 231), but convincing evidence in human gastric mucosa is lacking.

H. pylori could affect cell cycle by inducing gastric epithelial cells apoptosis and a compensatory gastric epithelial hyperproliferation (232). Also, *H.pylori* activates various pathways in cell cycle such as β-catenin pathway and EGF pathway (169). Increased turnover and DNA replication in this hyperproliferative environment leads to the emergence of metaplasia and dysplasia (172).

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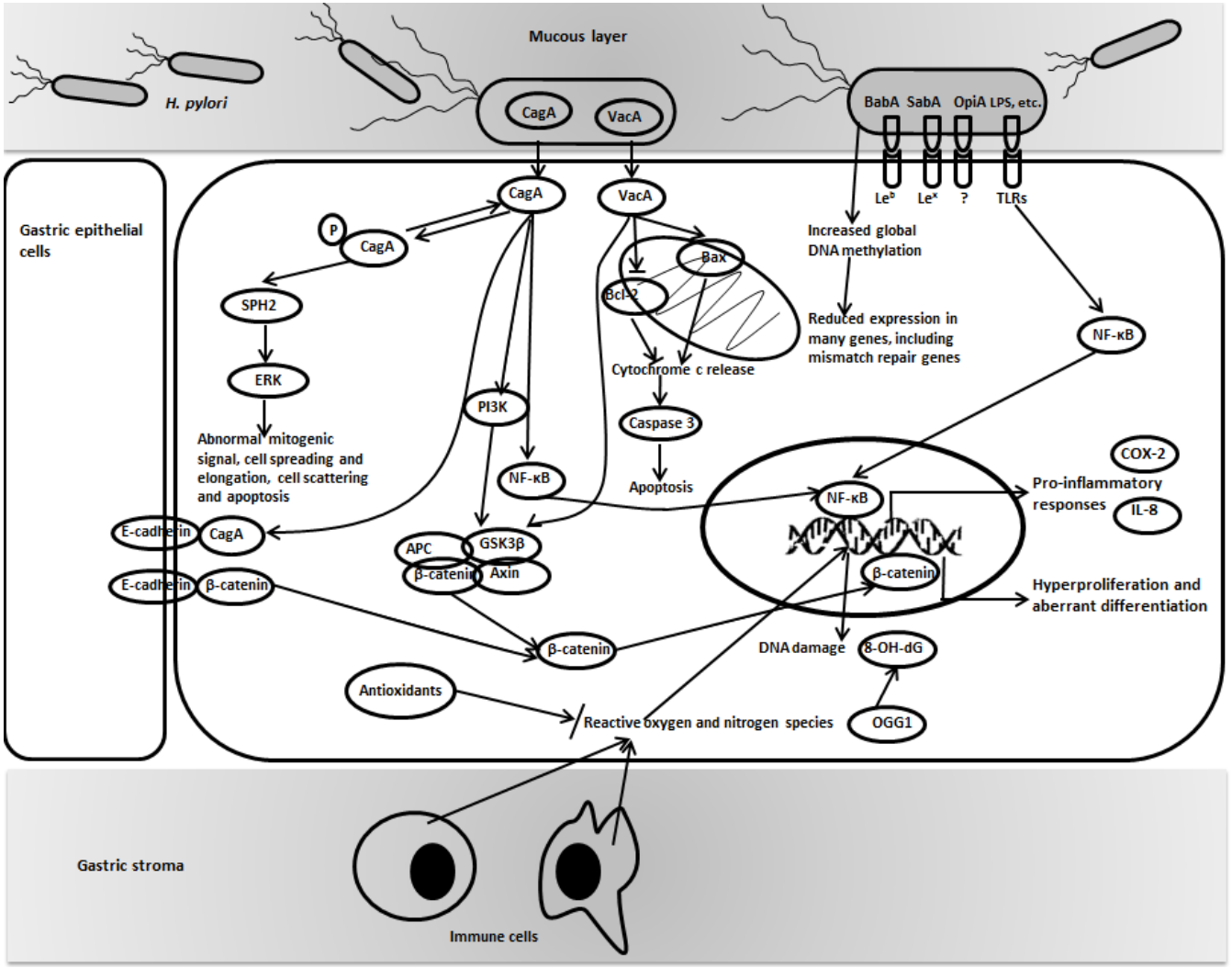
Hypochlorhydria induced by *H. pylori* infection is another potential mechanism linking *H. pylori* to gastric cancer (150, 172). Gastric acid secretion is inhibited by IL-1β

Figure 1.1. *H. pylori*-related gastric carcinogenesis



which is induced by *H. pylori* infection (150); *H. pylori* induced atrophy including loss of the specialized acid-secreting parietal cells also leads to hypochlorhydria (172). It is suggested that hypochlorhydria allows the spread of *H. pylori* induced inflammation from the antrum to the corpus and the growth of other bacteria found in the oral cavity or elsewhere that can facilitate the production of carcinogenic nitrosamines (150, 172).

Epstein-Barr Virus (EBV)

EBV has been suggested as a causative microorganism for gastric cancer (233-235). EBV-associated gastric cancer, comprising around 10% of all gastric cancer cases, consists of monoclonal growth of EBV-infected cells (235, 236). Histopathologically, EBV-associated gastric cancers consist of two subtypes: lymphoepithelioma-like carcinoma and the ordinary type of gastric cancer (234). Molecularly, EBV-associated gastric cancers have a higher global and non-random CpG island methylation level in the promoter region of many cancer-related genes (234). The proposed mechanism for hypermethylation was the activation of DNA methyltransferase 1 (DNMT1) by EBV latent membrane protein 2A (234, 236).

A few epidemiological studies investigated the association between EBV infection and gastric cancer with mixed results (237-240); two studies found that EBV seropositivity was positively associated with gastric cancer risk (237, 238), one study found negative association (239), and one study found null association (240).

Smoking

Smoking is another convincing risk factor for gastric cancer after *H.pylori* (150) and has been classified as carcinogenic to human stomach by IRAC since 2004 (241). In 1997, a meta-analysis (242) including 40 studies showed that compared with non-smokers, smoking was associated with 1.59-fold increased risk of gastric cancer among

males and 1.11 among females (p-value for difference, 0.04). The summary risk was higher in cohort studies than in case-control studies and higher in studies from North America than in other regions. A number of studies separated current and ex-smokers, and the relative risk was higher in current smokers (1.47) than in ex-smokers (1.18). Also, a dose-response was suggested.

In 2006, Nishino Y et al. systematically reviewed the epidemiologic evidence from 10 cohort studies and 16 case-control studies for the association of tobacco smoking and gastric cancer risk among Japanese population (243). The summary relative risk for current smokers was 1.56 (95% CI=1.36-1.80), 1.79 (95% CI=1.51–2.12), 1.22 (95% CI=1.07–1.38) for the total population, men and women, respectively.

In 2008, another meta-analysis was conducted for 42 cohort studies (244). Compared to never smokers, the summary relative risk for current smokers was 1.53 (95% CI= 1.42–1.65), 1.62 (95% CI= 1.50–1.75), 1.20 (95% CI= 1.01–1.43) for the total population, men and women, respectively. A dose response was found where the relative risk increased from 1.3 for the lowest consumptions to 1.7 for 30 cigarettes/day. Smoking was significantly associated with increased risks of cardia (RR = 1.87; 95% CI: 1.31–2.67) and non-cardia (RR = 1.60; 95% CI: 1.41–1.80) gastric cancers.

One pooled analysis consisted of 10 population-based case–control studies and 2 cohort studies specifically investigated the association of smoking with esophagogastric junctional adenocarcinoma (245). This study showed that cigarette smoking was associated with an increased risk of esophagogastric junctional adenocarcinoma (OR = 2.18, 95% CI = 1.84 - 2.58) which was similar among males and females. A dose-response for smoking and was found, and longer smoking cessation was associated with a decreased risk.

Recently, a meta-analysis including 33 studies up to January 2010 was conducted specifically for gastric cardia adenocarcinoma. Compared to never-smokers, the summary relative risk was 1.76 (95% CI=1.54 –2.01) for ever-smokers, 2.32 (95% CI=1.96 –2.75) for current smokers, and 1.62 (95% CI=1.40–1.87) for ex-smokers. A dose-response for pack-year and duration was found (246).

The mechanism linking smoking to gastric cancer is complex involving both genotoxic and non-genotoxic effects (247). There are 62 chemical compounds identified in tobacco that been classified as carcinogens for humans or animals by IARC (241, 248). Genotoxic carcinogens in cigarette smoke induce DNA damage due to gene point mutation, deletions, insertions, recombinations, rearrangements, and chromosomal aberrations. Two of the most abundant genotoxic agents in cigarette smoke are polycyclic aromatic hydrocarbons (PAHs) and nitrosamines (247). Besides genotoxic effects, non-genotoxic effects of cigarette smoke also play an important role in gastric carcinogenesis. These effects include alterations in cellular functions including cell proliferation and cell death (247).

Alcohol Drinking

Recently, Tramacere et al. conducted a meta-analysis on alcohol drinking and gastric cancer risk (249). They included 44 case–control and 15 cohort studies with 34,557 gastric cancer cases. Compared to non-drinkers, the summary relative risk was 1.07 (95% CI=1.01–1.13) for alcohol drinkers and 1.20 (95% CI=1.01–1.44) for heavy alcohol drinkers (≥4 drinks/day). The estimates were higher for noncardia gastric cancers (RR = 1.17, 95% CI 0.78–1.75) than for cardia gastric cancers (RR = 0.99, 95% CI 0.67–1.47). No significant differences were found across strata of sex.

Salt, Salty and Salted Food Consumption

In 2007, the World Cancer Research Fund / American Institute for Cancer Research listed salt, salty and salted food consumption as a “probable” risk factor to increase gastric cancer risk (250).

In 2009, Wang et al. extensively review the epidemiological studies of salt and gastric cancer risk (251), and they concluded that “the majority of ecological studies indicate that the average salt intake in each population was closely correlated with gastric cancer mortality. Most case-control studies showed similar results, indicating a moderate to high increase in risk for the highest level of salt or salted food consumption. The overall results from cohort studies are not totally consistent, but are suggestive of a moderate direct association.” Among the sixteen case-control studies that investigated overall dietary salt or sodium intake, eight of them reported strong statistically significant increases in risk for gastric cancer (OR = 1.5-5.0 for the highest intake levels), seven of them reported statistically non-significant OR of 1.1 to 1.5 for consumption above the median intake, and the remaining study reported no association (251).

Very recently, a meta-analysis of 7 prospective studies (268,718 participants, 1,474 events, follow-up 6-15 years) on salt intake and risk of gastric cancer was conducted (252). Compared to “low” salt intake, the summary relative risks of gastric cancer were 1.68 (95% CI=1.17-2.41) and 1.41 (95% CI=1.03-1.93) for “high” and “moderately high” salt intake, respectively.

Another meta-analysis summarized salt intake and risk of gastric intestinal metaplasia which is a precancerous lesion for gastric cancer (253). A total of 17 studies were included, and the OR was 1.68 (95% CI = 0.98–2.90) for the association between salted/salty meat and intestinal metaplasia and the OR was 1.53 (95% CI = 0.72–3.24) for salt preference, respectively.

Several mechanisms by which salt intake increases gastric cancer risk have been proposed. High dietary salt intake may facilitate the colonization of *H.pylori* (254) by increasing surface mucous cell mucin and decreasing gland mucous cell mucin (255) and potentiate *H.pylori*-associated carcinogenesis by inducing proliferation, pit cell hyperplasia and glandular atrophy (254). At molecular level, high salt concentrations up-regulate the expression of *H. pylori* CagA, leading to an increased amount of CagA translocated into gastric epithelial cells and an enhanced ability of *H. pylori* to alter gastric epithelial cell function (256). Another possible explanation is that high dietary salt intake changes the mucous viscosity, potentiates exposure to carcinogens such as N-nitroso compounds, and leads to cell death (257). Other possible explanations include increased damage, inflammatory response (258), and induction of hypergastrinemia (255).

Vegetables and Fruits

In 2007, the World Cancer Research Fund / American Institute for Cancer Research listed non-starchy vegetables, allium vegetables, and fruits consumption as a "probable" risk factor to decrease gastric cancer risk (250).

Non-starchy Vegetables: According to the review by the World Cancer Research Fund / American Institute for Cancer Research (250), most estimates from cohort studies were close to one; a summarized effect estimate of 0.98 (95% CI 0.91–1.06) per 100 g/day was given by a meta-analysis on 9 independent estimates from 7 cohort studies (250). Another meta-analysis (259) based on 8 articles took duration of follow-up into account and gave an overall RR of 0.88 (95% CI = 0.69–1.13) using incidence studies and 0.71 (95% CI = 0.53–0.94) when only considering incidence studies with the longer follow-up ($\geqslant$10 yr), suggesting the importance of the duration of follow-up.

Evidence from case-control studies is more consistent and convincing (250). Of the 45 case-control studies, 28 reported statistically significant decreased risks and the majority of the 17 remaining studies with no significant effect on risk were in the direction of reduced risk. Meta-analysis on possible 20 studies reported an overall effect estimate of 0.70 (95% CI 0.62–0.79) per 100 g/day.

Allium vegetables: A recent meta-analysis (260) including 19 case-control and 2 cohort studies with a total of 543,220 subjects showed that in comparison to the lowest consumption groups, highest consumption of allium vegetables was associated with a reduced risk for gastric cancer (OR, 0.54; 95% CI, 0.43–0.65).

Fruits: As reviewed by the World Cancer Research Fund / American Institute for Cancer Research (250), 10 cohort studies reported reduced risks (statistically significant in one) for the highest consumption group in comparison to the lowest and 6 reported increased risks (statistically significant in one). Meta-analysis which was possible on 8 studies gave an overall effect estimate of 0.95 (95% CI 0.89–1.02) per 100 g/day. Another meta-analysis (259) showed that when the duration of follow-up was longer (>=10 yr), the effect was stronger. Meta-analysis on 26 case-control studies gave an overall effect of 0.67 (95% CI 0.59–0.76) per 100 g/day.

Chemoprevention Trials with Antioxidative Vitamins: Given the consistent negative associations of vegetables and fruits with gastric cancer risk from observational studies, investigators tried to reduce gastric cancer risk with antioxidative vitamins, presumably the active ingredients in vegetables and fruits which are responsible for the protective effects. As summarized in **Table 1.4**, eight studies have been conducted and two of them found favorable effects, four showing no effect and two showing slightly increased risk (261-268).

Table 1.4. Summary of the chemoprevention trials with antioxidative vitamins for gastric cancer prevention.

Author	Year	Place	Sample size	Intervention	Duration	Endpoint	Effects
Blot W (273)	1993	China	331GC cases /29,584	β-carotene, VE, selenium (combined)	About 5 years	Incidence	RR = 0.79; 95% CI = 0.64-0.99)
Correa P (274)	2000	Colombia	976	ascorbic acid, β-carotene	About 3 years	Evolution of precancerous lesions	About 4-fold increased chance of regression for both
Zhu S (275)	2003	China	216	β-carotene	About 2 years	Evolution and incidence	No effect
Malila N (276)	2002	Finland	126 gastric cancer cases /29,133	α-Tocopherol, β-carotene	About 6 years	Incidence	α-Tocopherol: RR=1.21 (0.85-1.74); β-carotene: RR=1.26 (0.88-1.80)
Hennekens CH (277)	1996	USA	40 gastric cancer cases /11,035	β-carotene	About 12 years	Incidence	No effect
Li JY (278)	1993	China	77 gastric cancer cases /3318	14 vitamins, 12 minerals	About 6 years	Incidence	RR=1.18 (0.76-1.85)
You WC (279)	2006	China	3365	Vitamin C, E, and selenium	About 7 years	Evolution	No effect
Plummer M (280)	2007	Venezuela	1980	Vitamin C, E, and β-carotene	About 3 years	Evolution	Regression rate ratio = 1.09 (95% CI = 0.90 to 1.33)

The Gastric Precancerous Cascade

It is well accepted that the intestinal type of gastric adenocarcinomas is preceded by a prolonged precancerous process [Correa's model (2-4, 66) in which gastric mucosa progresses from “normal gastric mucosa → superficial gastritis (later renamed non-atrophic gastritis, NAG) → multifocal atrophic gastritis (MAG) without intestinal metaplasia → intestinal metaplasia (IM) of the complete (small intestine) type → IM of the incomplete (colonic) type → low-grade dysplasia (low-grade noninvasive neoplasia) → high-grade dysplasia (high grade noninvasive neoplasia) → invasive adenocarcinoma”.

According to the Correa's model (2-4, 66), in normal gastric mucosa, only small numbers of scattered mononuclear inflammatory cells are present, and as a consequence of Helicobacter pylori (*H. pylori*) infection, the lamina propria is infiltrated with increased mononuclear leukocytes (chronic inflammation) and polymorphonuclear neutrophils (acute inflammation) which characterizes gastritis. At the initial stage, gastritis is non-atrophic without loss of normal glandular tissue, and depending on the presence of virulent factors (cag-positive and vacA s1m1) in the infecting *H. pylori* strain, NAG can progress to MAG with loss of normal glandular tissue following chronic inflammation. IM is viewed as an advanced stage of atrophy where the gastric gland is replaced by the intestinal gland and is classified into two types: the small intestine or complete type, and the colonic or incomplete type based on morphology and enzyme histochemistry. Recent evidence suggested that IM is the origin of dysplasia (269) which is also called intraepithelial neoplasia or noninvasive neoplasia. Dysplasia displays neoplastic phenotype where the cells are enlarged, hyperchromatic, with crowded nuclei and within the bounds of the basement membrane, and the dysplastic glands are irregular in shape.

The prevalence of gastric precancerous lesions is high in high-risk areas. One report from Linqu County, a high gastric cancer risk area in China, with 3433 residents aged 35 to 64 yr (270) showed that chronic atrophic gastritis was nearly universal; normal mucosa or only superficial gastritis was only found in less than 2% of the study population. The prevalences of intestinal metaplasia and dysplasia in Linqu were 33% and 20%, respectively, compared to 7.9% and 5.6% in Changshan, a nearby low risk area in China (271). Another report from Venezuela showed that the prevalences for chronic gastritis, atrophic gastritis, intestinal metaplasia, and dysplasia were 49.0%, 14.2%, 26.7%, and 5.8% respectively among participants in a gastric cancer screening program who were between 35 and 69 years of age and in general good health (272). One study from a high gastric cancer risk area in Iran found that mucosal atrophy was found in 39.3% of antral and 21.9% of cardia samples, intestinal metaplasia in 8.7% of antral and 3.8% of cardiac biopsies, and dysplasia in 0.2% of antral and 0.3% of cardiac biopsies (273). In the United States, it was reported that among the individuals who attended gastrointestinal services in New Orleans, Louisiana, 10.6% of Caucasians had IM or dysplasia, and 17.2% of African Americans had IM or dysplasia (274).

Individuals with gastric precancerous lesions are at higher risk of gastric cancer. One cohort study conducted in China with five-year follow-up (275) showed that compared with subjects with superficial gastritis (SG) or chronic atrophic gastritis (CAG) at baseline, the risk of gastric cancer was elevated by 17.1 times for those with baseline diagnoses of superficial IM, 29.3 times for those with deep IM or mild dysplasia or IM with glandular atrophy and neck hyperplasia, and 104.2 times for those with moderate or severe dysplasia. Also studies from other regions showed increased gastric cancer risk among patients with IM or dysplasia (276-280).

Screen and Early Detection for Gastric Cancer and Precursors

Despite the advances in treatment, five-year survival for gastric cancer patients is poor Japan had the highest five-year survival of 52% possibly due to the mass screening by photofluoroscopy that has been practiced since the 1960s, and the lowest rate was observed in sub-Saharan Africa (6%) (24). According to data from the SEER (30), the five-year survival is much higher among cases diagnosed with localized gastric cancer compared to cases diagnosed at a late state (61.4% versus 3.6%), and this suggests the importance of early detection. However, screening for gastric cancer is not commonly practiced, and except Japan and Korea where gastric cancer is highly prevalent, there is no national guidelines or recommendations for gastric cancer screen in many countries including the United States.

Several screening methods for the early detection of gastric cancer have been proposed, including barium-meal photofluorography, gastric endoscopy, serum pepsinogen test, gastrin-17 test and *H.pylori* antibody test, although none of has been assessed by randomized clinical trials. According to an evaluation of different methods, screening using barium-meal photofluorography was recommended for both population-based and opportunistic screening, while the other methods were not recommended for population-based screening due to insufficient evidence (281).

Barium-meal Photofluorography: Trained radiographic technicians take the photofluorogram, and a suspected abnormality (such as decreased calibre of lumen, stenosis, deformity, rigidity, indentation, the presence of a niche or a filling defect in the wall, flattening of the randwall, barium pooling, irregularity in the gastric area, change in gastric fold, or presence of polypoid lesion) is referred to further diagnostic examinations that include full-size radiography, endoscopy, and biopsy (282).

Photofluorography screening for gastric cancer has been conducted in Japan since 1960 (283), and there was a concurring decrease in incidence and mortality from gastric cancer. However, no randomized studies have been conducted to investigate its effectiveness. Observational studies showed screening with photofluorography for gastric cancer was associated with a decrease of about 40-60% of gastric cancer mortality (284).

The sensitivity and specificity of photofluorography screening for gastric cancer were assessed using cancer registry data by a number of studies and summarized by Hamashima et al. (281). The sensitivity ranged from 60% to 80% and the specificity from 80% to 90% (281), and the highest accuracy was reported by Murakami et al with sensitivity of 88.5% and specificity of 92.0% (285). The 5-year survival rate ranged from 74% to 80% for the screened group and from 46% to 56% for the non-screened group in whom cancer was detected in the clinical setting (281). Harms of gastric cancer screening using radiography include exposure to radiation, false swallowing of barium meal (frequency: 0.08 to 0.17%), defecation delay (frequency: 4–11%), constipation ileus, and false negative with rate ranging from 10% to 30% (281).

Endoscopy: Endoscopy has attracted more and more attention because its high detection rate especially for superficial flat and non-ulcerative lesions that barium-meal photofluorography can miss (282). One study compared different screening techniques in Japan and concluded that endoscopy is superior for the detection of early gastric cancer compared with direct X-ray and mass screening program with photofluorography. The cancer detection rate with endoscopy was approximately 2.7 and 4.6 times higher than direct X-ray and mass screening program with photofluorography, respectively (286).

Two studies reported the accuracy of endoscopy. The first study reported a sensitivity of 77.8% based on 3-year follow-up using the cancer registry system in Fukui prefecture among symptomatic patients, but no specificity was reported (287). The second study was based a follow-up survey of individual participants, and the sensitivity was reported to be 84.0% (288).

In terms of mortality reduction, there was one cohort study conducted in Linqu County, an area with a high incidence of gastric cancer, China (289). Among 4,394 residents screened using endoscopy between 1989 and 1999, 37 gastric cancer deaths were observed between 1989 and 2000, yielding a standardized mortality ratio of 1.01 (95% CI 0.72-1.37) for the entire cohort, 1.13 (95% CI 0.77-1.57) for males, and 0.65 (95% CI 0.26-1.32) for females. Harms associated with endoscopy include pharynx anesthetic sedation, bleeding or perforation (frequency: 0.012%), and false negative of about 16% (281). Despite its high detection rate, it is not feasible to perform mass screening by endoscopy even in highly developed countries such as Japan due to the lack of skilled endoscopists and availability of gastroscopy (282).

Serum Pepsinogen Test: Serum pepsinogen test is a popular non-invasive serological screening for gastric cancer especially in Japan due to no significant associated harms and the high expenses and logistic burdens of invasive screening tests. Pepsinogen, released specifically in the stomach, is the precursor of pepsin that is one of the three principal protein-degrading enzymes. Hydrochloric acid (HCl), which is released from parietal cells in the stomach lining, activates pepsinogen into pepsin. When food is ingested, the hormone gastrin and the vagus nerve prompt the stomach lining to release both pepsinogen and HCl.

There are two isoforms of pepsinogen, PGI (Also called PG“A”) and PGII (PG“C”), with different biochemical and immunological properties (290, 291). The distribution of cells that release PGI and PGII had been clearly identified by immunohistochemistry using specific antibodies or in-situ hybridization (292-294). PGI is produced by chief and mucous neck cells in fundic gland mucosa where acid-secreting glands locate; however, PGII is produced not only in these cells, but also in the cardiac, pyloric, and duodenal Brunner gland cells, and the distribution of PGII producing cells spreads from the entire stomach to the duodenum. Intestinal metaplastic cells usually do not secrete pepsinogen, but dysplastic and carcinoma cells express pepsinogen (mostly PGII)(4). Also, PGII is stimulated by inflammation (such as *H. pylori* induced inflammation) and cell proliferation (2). While majority of pepsinogen enters into the stomach lumen, about 1% of the total is secreted into the blood stream by unknown mechanism (295).

Gastric cancer, especially the intestinal type, is the end result of progression of precancerous lesions including multifocal atrophic gastritis, intestinal metaplasia, and dysplasia driven by *H. pylori* induced inflammation (2-4, 66). As chronic atrophic gastritis proceeds, mucosal atrophy spreads from the pyloric gland up to fundic gland, resulting a stepwise decrease in PGI levels and PGI/PGII ratios since PGII remains fairly constant (295-297). One study in China suggested that the PGI/PGII ratio decreased monotonically along the sequence of superficial gastritis, chronic atrophic gastritis, intestinal metaplasia, dysplasia, and stomach cancer (298).

Several cut points for PGI and PGI/PGII ratio exist, and the most commonly used ones in Japan are PGI ≤ 70 ng/ml and PGI/PGII ratio ≤ 3.0. A pooled analysis from Japan included 300,000 participants from 27 population based screening studies (n= 296,553) and 15 selected groups (n=4,385) (296, 299). Based on the results from the

population based screening studies, PGI ⩽ 70 ng/ml and PGI/PGII ratio ⩽ 3.0 yield a sensitivity of 77% and specificity of 73% to detect gastric cancer (296, 299); the positive predictive value ranged from 0.77% to 1.25%, but the negative predictive value was above 99.08% (296, 299). Among the selected groups, the pooled sensitivity is 57% and specificity of 80%. In terms of detection of dysplasia, PGI level ⩽ 50 ng/ml and PGI/PGII ratio ⩽ 3 obtained a sensitivity of 65% and specificities varying from 74% to 85%, both with NPV > 95% (296, 299). For identification of atrophic gastritis, since included studies used different cut off points for the PG test, the authors did not pool the results; the range of sensitivity is from 18.8% to 98.5% and specificity from 64% to 100%.

To date, a total of 18 prospective studies (including eight prospective cohort studies and the nested case-control studies) have investigated whether PG test including PGI, PGII and the ratio could predict gastric cancer risk (**Table 1.5**). The results consistently showed that lower PGI level or the ratio of PGI to PGII was associated with a 3-4 fold increased risk of gastric cancer with a dose-response relationship and associations being stronger for the intestinal type of gastric cancer and non-cardia gastric cancer. Studies also found that higher PGII level was associated with increased gastric cancer risk (300, 301), especially the diffuse type (302-304). In addition, one study analyzed the sensitivity and specificity of PG test for gastric cancer occurrence in a prospective setting and they found that the most predictive sPG test criteria were PGI ⩽ 59 ng/ml and PGI/PGII ratio ⩽ 3.9 with a sensitivity of 71.0 and specificity of 69.2% (305).

Table 1.5. Summary of the prospective studies investigating the association of baseline PG with gastric cancer risk

Author	Year	Study Design	Sample size	Biomarker measures	Duration	Results
Pastore JO (306)	1972	Cohort study	112cases/6859 Japanese	Pepsins High: >= 500 ug/mL middle: 200-499 low: < 200	10 yrs	Incidence High: 1.8% middle: 1.4% low: 4.9% Stronger among men.
Nomura AM (307)	1980	Nested CC study	58 cases, 96 matched controls from 7498 Japanese	PG1 High: >= 20 ng/mL Low: < 20 ng/mL	44.5 mths	Low **PG1** in 15/48 cases and 6/96 controls Only for intestinal type
Stemmermann GN (300)	1987	Nested CC study	87 cases, 250 controls from 7498 Japanese men in Hawaii	PG1, PG2, PG1/PG2	12 yrs	**PG1:** Low (<20 ng/ml) vs. >=80: OR=8.73 (95% CI 3.60-21.15) with trend **PG2:** Low (<14 ng/ml) vs. >=26: OR=0.69 (95% CI 0.34-1.38) with trend **Ratio:** Low (<1.30) vs. >=3.50: OR=9.72 (95% CI 4.18, 22.56) with trend Only for intestinal type
Parsonnet J (308)	1993	Nested CC study	136 cases (carida and non-carida) and 136 controls from 128,992 Americans	PG1, PG2	20 yrs	**PG1:** Low (<50 ng/ml) vs. >=125: OR=3.8 (95% CI 1.0-13.5) with trend Stronger for non-cardia GC. Interaction between PG1 and anti-*H.pylori* IgG (Without HP, PG1 is not associated with GC)
Aromaa A (309)	1996	Nested CC study	84 cases, and 146 controls from 39,268 Finnish	PG1 Low: <49 mg/L	13 yrs	Low **PG1**: 2.68 (95% CI 1.35-5.30)
Watanabe Y (310)	1997	Nested CC study	45 cases, and 225 controls from 2858 Japanese	PG1 PG2 PG1/PG2	8 yrs	**PG1:** Low (<=70 ng/ml) and **Ratio:** Low (<3.00) together: OR=3.38 (95% CI 1.54, 7.42).
Ohata H (311)	2004	Cohort study	45cases/4655 Japanese	PG1, PG2, PG1/PG2	7.7 yrs	**CAG defined as: PG1:** Low (<=70 ug/L) and **Ratio:** Low (<3.00): HR: 3.03 (95% CI 1.67, 5.49). Interaction between CAG and anti-*H.pylori* IgG

Watabe H (312)	2005	Cohort study	43cases/6983 Japanese	PG1, PG2, PG1/PG2	4.7 yrs	**CAG defined as: PG1:** Low (<=70 ug/L) and **Ratio:** Low (<3.00): RR: 8.26 (95% CI 4.32, 15.79) (Calculated by myself using data from the paper.) HR: 6.2 (95% CI 2.9–13) from a follow-up paper based on the same study population. (Helicobacter. 2009 Apr;14(2):81-6) Interaction between CAG and anti-*H.pylori* IgG
Sasazuki S (313)	2006	Nested CC study	511cases, 511 matched controls From 123,576 Japanese	PG1, PG2, PG1/PG2	14 yrs	**CAG defined as: PG1:** Low (<=70 ug/L) and **Ratio:** Low (<3.00): 3.8 (95% CI 2.7, 5.4) with trend. Interaction between CAG and anti-H.pylori IgG
Knekt P (314)	2006	Nested CC study	225 cases and 435 controls from 39,268 Finnish	PG 1	24 yrs	**PG1:** Low (<49 ug/L) OR: 2.24 (95% CI 1.43, 3.49) for noncardia cancers, OR: 1.85 (95% CI 0.55, 6.16) for cardia cancers. Interaction between CAG and anti-H.pylori IgG and IgA
Oishi Y (315)	2006	Cohort study	89 cases/2446 Japanese	PG1, PG2, PG1/PG2	14 yrs	**CAG defined as: PG1:** Low (<=70 ug/L) and **Ratio:** Low (<3.00): **Men:** HR: 3.42 (1.92, 6.11) **Women:** HR: 1.88 (0.69, 5.16)
Hansen S (316)	2007	Nested CC study	129 non-cardia and 44 cardia cancers, and 3 matched controls for each case from 101,601 norwegian	PG1, PG2, PG1/PG2	11.9 yrs	**Ratio<2.5:** OR: 4.47 (95% CI 2.71, 7.37) for noncardia cancers, OR: 1.60 (95% CI 0.62, 4.14) for cardia cancers with trend. Interaction between CAG and anti-H.pylori IgG
Yanaoka K (302)	2008	Cohort study	63 cases/5209 Japanese men	PG1, PG2, PG1/PG2	10 yrs	**CAG defined as: PG1:** Low (<=70 ug/L) and **Ratio:** Low (<3.00): PG I <=70 and PG I/II <3.0: HR 3.60 (95% CI 2.17-5.96) PG I <=50 and PG I/II <3.0: HR 4.55 (95% CI 2.62-7.43) PG I <=30 and PG I/II <2.0: HR 5.16 (95% CI 2.77-9.51)
Ren JS (317)	2008	Nested CC study	330 non-cardia and 546 cardia, and 974 controls from 29, 584 Chinese	PG1, PG2, PG1/PG2 (Assessed by Biohit) (Also, considered non-linear associations)	15 yrs	Non-cardia **PG1** <=50 ug/L: HR=1.87 (95% CI 0.98 to 3.56) **PGI/II ratio** <= 3: HR=2.17 (95% CI 1.26 to 3.74) Cardia **PG1** <=50 ug/L: HR=1.74 (95% CI 0.99 to 3.06) **PGI/II** ratio <= 3: HR=1.58 (95% CI 0.95 to 2.63)

Abnet CC (301)	2011	Nested CC study	141 cases, 282 controls from 73,222 women	PG1, PG2, PG1/PG2 (Assessed by Biohit) (Also, considered non-linear associations)	6 yrs	**PG1** <=50 ng/mL: OR=4.23 (95% CI 1.86 to 9.63) **PG2** >=6.6 ng/mL: OR=3.62 (95% CI 0.98 to 3.56) **PGI/II** ratio <= 4: HR=1.60 (95% CI 0.79 to 3.22)
Shikata, K (305)	2012	Cohort study	69 cases/2446 Japanese	PG1, PG2, PG1/PG2	10 yrs	**PG1** <=59 ng/mL and **PGI/II** ratio <= 3.9 HR=5.08 (95% CI 2.93 to 8.81) Interaction between CAG and anti-H.pylori IgG
Zhang XH(318)	2012	Cohort study	26 cases /1501 Chinese	PG1, PG2, PG1/PG2 (Assessed by RAD)	14 yrs	**CAG defined as:** PG1: Low (<=70 ug/L) and Ratio: Low (<3.00): **OR:** 4.23 (95% CI 1.92–9.34) Interaction between CAG and anti-H.pylori IgG
Lomba-Viana R (319)	2012	Cohort study	9 cases/514 portuguese	PG1, PG2, PG1/PG2	3 yrs	**CAG defined as:** PG1: Low (<=70 ug/L) and Ratio: Low (<3.00): **RR:** 1.75 (95% CI 0.44, 6.93) (Calculated by myself using data from the paper.)

Serum Gastrin-17: Gastrin, released by G cells in the antrum, is a hormone that stimulates gastric acid and pepsinogen secretion and the growth of the gastric mucosa (320). As a result of the cellular posttranslational maturation process of progastrin, the G cells released a mixture of different forms of gastrin into the circulation including gastrin-71, -52, -34, -17, -14, and -6 (320). The dominant forms in healthy human serum are gastrin-34 and -17, while the major and potent tissue form in healthy antral mucosa is G-17 which is almost exclusively produced by the antrum G-cells (321). Various factors including dietary protein stimulus and low acidity in the stomach stimulate the secretion of G-17 from the G cells and gastrin (322). The decline of theG-17 levels is positively correlated with the degree of atrophy in the antrum (323, 324). A low G-17 level is an indication of advanced atrophic gastritis in the gastric antrum (325), which is associated with increased risk of gastric cancer and peptic ulcer disease (323, 324, 326-331). However, as pointed by Correa P (332), G-17 as an biomarker for atrophy in the antrum needs more investigation given that G - 17 is unstable in serum and low sensitivity.

On the other hand, abnormally high G-17 levels can be used as a biomarker of hypo- or achlorhydria induced by atrophic gastritis that is limited to the gastric corpus where acid-secreting glands locate (330, 331). Also, G-17 levels increases substantially with gastric cancer (282, 331, 333-335) while some studies showed no increase (336). However, it is commonly accepted that the use of serum gastrin-17 alone, which reflect the distal stomach status, cannot be used as a single serum marker of gastric cancer (282).

To date, only one prospective case-control study (376 controls and 129 cases) has investigated whether serum G-17 level could predict gastric cancer risk (316). In this study, they found that compared to individuals in the first quintile (serum G17 level:

2-20 ng/L), individuals in the higher quintiles have increasing risk of gastric cancer, and the association is stronger for non-cardia gastric cancer and the diffuse type of gastric cancer.

Serum Anti Helicobacter pylori (*H. pylori*) IgG: The presence of IgG antibody indicates current or past infection. Serum IgG titers decrease slowly after the eradication of the organism and the test may turn negative after one year of the eradication (337).

Regardless of the strong immune responses, *H. pylori* will not be eradicated until being treated with a combination including antibiotics, and usually chronic gastritis will subsequently develop (225, 226). Indeed, the ineffective humoral response against *H. pylori* may contribute to pathogenesis. For example, monoclonal antibodies against *H. pylori* could cross-react with human gastric epithelium and induce gastritis (338, 339). Some studies found that higher levels of serum *H. pylori* IgG were associated with higher *H. pylori* density (340-342) and more severe gastric inflammation (341, 343-345). Therefore, humoral immune responses to *H. pylori* infection might represent more of a marker of infection than an indicator of protection.

The sensitivity and specificity of serum anti *H.pylori* IgG test for *H.pylori* infection were reported to be varying from 90 to 93 and from 95 to 96, respectively (337). In terms of discrimination of normal gastric mucosa from the diseased, according to unpublished data with 15,953 subjects over 15 years from the same study population as the dissertation research, the sensitivity and specificity are 66.8% and 72.1%, respectively. The mean levels among patients with normal mucosa (n=1751), superficial gastritis (n=3922), gastric erosion and ulcer (n=975), atrophic gastritis (n=3018), dysplasia (n=337) and gastric cancer (n=97) were 8.7, 29.0, 39.0, 39.3, 31.0, and 27.1

with the peak at atrophic gastritis, respectively. Another study from China found a similar pattern where the prevalence of H.pylori infection determined by Anti H.pylori IgG level peaked among patients with severe atrophic gastritis (346).

Consistent with our findings that *H. pylori* seropositivity and antibody titers were lower in those with gastric cancer than in those in any of the other gastric histopathologies, in a cross-sectional study of 10,234 endoscoped Japanese, Yamaji *et al* found that the prevalence of gastric cancer was higher among those who were 'weakly positive' than those 'strongly positive' (0.51% vs. 0.47%) (347). Also, two nested case-control studies have investigated the association between different levels of IgG titer and gastric cancer risk. In the first study with 350 gastric cancer cases and 350 matched controls (348), IgG titer was divided into four grades: negative (<10), low (10.0-32.8), middle (32.9-54.6) and high (≥ 54.7), and the authors found that using the individual with negative IgG titer as the reference group, those with low IgG titer had the highest risk for intestinal type of gastric cancer (OR: 5.9, 95% CI: 3.0 - 11.6) compared to those with middle or high IgG titers; while for diffuse type of gastric cancer, those with high IgG titer had the highest risk (OR: 7.8, 95% CI: 2.4 - 24.9) compared to those with middle or high IgG titers. In the second study with 511 gastric cancer cases and 511 matched controls, the authors also found that low IgG titers was associated with highest risk and the association was stronger among those with severe mucosal atrophy which was assessed by PG test (349).

Combination of multiple methods: As reviewed above, every method has its own advantages and disadvantages and reflects different aspects of the gastric carcinogenesis, so it is appealing to combine multiple methods to improve sensitivity, specificity and risk prediction.

Serum Pepsinogen Test and Serum Anti H. pylori IgG

This combination is the most common one, and eight studies have investigated the usefulness of this combination to predict gastric cancer risk and the results consistently showed that the combination can improve risk prediction (**Table 1.6**). The highest risk was observed either among those who were *H. pylori* negative and PG test positive (305, 311, 313, 316, 318) or among those who were *H. pylori* positive and PG test positive (308, 312, 314).

Serum Pepsinogen Test and gastrin-17 test

In a study with 122 early gastric cancer cases and 178 gastric cancer free controls, a combination of low PG levels (PGI level <= 70 ng/ml and PGI/PGII ratio <= 3) and low gastrin-17 level has a sensitivity of 12.3% and a specificity of 98.9% to distinguish gastric cancer cases from controls (350). Also, the results suggested low PG levels and gastrin-17 level discriminated multifocal atrophic gastritis from other types of gastritis, and serologically defined multifocal atrophic gastritis was associated with 27 times higher risk of gastric cancer (350).

Serum Pepsinogen test, Gastrin-17 test and H. pylori Antibody Test

In 2002, Sipponen *et al* have proposed combining PGI, gastrin-17, and *H.pylori* antibody using an empirical algorithm to diagnose atrophic gastritis (323, 351). In the initial case-control study in Finland where the algorithm was first proposed, cases were 56 selected dyspeptic outpatients with advanced (moderate or severe) atrophic gastritis and controls were 44 outpatients without advanced atrophic gastritis (323). By using the algorithm, 81% of the patients were classified into the correct “gastritis categories” diagnosed by pathohistology. For discriminating atrophic gastritis

Table 1.6. Summary of the prospective studies investigating the interaction between baseline PG level and anti-H.Pyloir IgG on gastric cancer risk.

Author	Year	Study Design	Sample size	Biomarker measures	Duration	Results
Parsonnet J (308)	1993	Nested CC study	136 cases (carida and non-carida) and 136 controls from 128,992 Americans	PG1, PG2, IgG	20 yrs	**PG1:** Low (<50 ng/ml) HP(-) and PG1 high: 1 HP(+) and PG1 high: OR: 2.4 (95% CI 1.0-5.6) HP(-) and PG1 low: OR: 0.8 (95% CI 0.1-4.4) **HP(+) and PG1 low: OR: 10.0 (95% CI 3.3-30.5)**
Ohata H (311)	2004	Cohort study	45cases/4655 Japanese	PG1, PG2, PG1/PG2, IgG	7.7 yrs	**CAG defined as: PG1:** Low (<=70 ug/L) and **Ratio:** Low (<3.00): HP(-) and CAG(-): 1 (no cancer developed in this group) HP(+) and CAG(-): HR: 7.13 (95% CI 0.95-53.33) **HP(-) and CAG(+):HR: 61.85 (95% CI 5.60-682.64)** HP(+) and CAG(+):HR: 14.51 (95% CI 1.96-107.70) Similar trend for intestinal and diffuse type.
Watabe H (312)	2005	Cohort study	43cases/6983 Japanese	PG1, PG2, PG1/PG2, IgG	4.7 yrs	**CAG defined as**: **PG1**: Low (<=70 ug/L) and **Ratio**: Low (<3.00): HP(-) and CAG(-): 1 HP(+) and CAG(-): HR: 4.2 (95% CI 2.2-8.0) HP(-) and CAG(+):HR: 4.9 (95% CI 2.0-12.1) **HP(+) and CAG(+):HR: 10.1 (95% CI 5.6-18.2)**
Sasazuki S (313)	2006	Nested CC study	511cases, 511 matched controls From 123,576 Japanese	PG1, PG2, PG1/PG2, IgG	14 yrs	**CAG defined as: PG1:** Low (<=70 ug/L) and **Ratio:** Low (<3.00): HP(-) and CAG(-): 1 HP(+) and CAG(-): HR: 1.1 (95% CI 0.4-3.4) **HP(-) and CAG(+):HR: 8.2 (95% CI 3.2-21.5)** HP(+) and CAG(+):HR: 6.0 (95% CI 2.4-14.5)
Knekt P (314)	2006	Nested CC study	225 cases and 435 controls from 39,268 Finnish	PG 1, IgA, IgG	24 yrs	**PG1:** Low (<49 ug/L) IgA(-), IgG(-), CAG(-): 1 IgA(-), IgG(+), CAG(-): HR: 1.33 (95% CI 0.52-3.41) IgA(+), IgG(-), CAG(-): HR: 0 IgA(+), IgG(+), CAG(-): HR: 5.59 (95% CI 2.41-13.0) IgA(-), IgG(-), CAG(+): HR: 5.45 (95% CI 1.59-18.6) IgA(-), IgG(+), CAG(+): HR: 6.77 (95% CI 1.83-25.1)

						IgA(+), IgG(-), CAG(+): HR: 3.35 (95% CI 0.31-35.9) **IgA(+), IgG(+), CAG(+): HR: 10.9 (95% CI 4.31-27.7)**
Hansen S (316)	2007	Nested CC study	129 non-cardia and 44 cardia cancers, and 3 matched controls for each case from 101,601 norwegian	PG1, PG2, PG1/PG2, IgG	11.9 yrs	**CAG defined as Ratio<2.5** OR=3.45 (95% CI 2.01 - 5.91) among H.pylori seropositive **OR=12.6 (95% CI 2.25 - 70.7) among H.pylori seronegative**
Shikata, K (305)	2012	Cohort study	69 cases/2446 Japanese	PG1, PG2, PG1/PG2	10 yrs	**CAG defined as PG1** <=59 ng/mL and **PGI/II** ratio <= 3.9 HR=4.41 (95% CI 2.45 - 7.93) among H.pylori seropositive **HR=12.37 (95% CI 3.19 - 48.05) among H.pylori seronegative**
Zhang XH(318)	2012	Cohort study	26 cases /1501 Chinese	PG1, PG2, PG1/PG2 (Assessed by RAD)	14 yrs	**CAG defined as:** PG1: Low (<=70 ug/L) and Ratio: Low (<3.00): HP(-) and CAG(-): 1 HP(+) and CAG(-): RR: 8.67 (95% CI 1.15-65.62) HP(-) and CAG(+):RR: 23.15 (95% CI 2.05-260.89) **HP(+) and CAG(+):RR: 27.47 (95% CI 3.35-225.42)**

in general from non-gastrophic gastritis or normal stomach, this combination had a sensitivity of 89% and specificity of 93%.

Serum Pepsinogen Test, Gastrin-17 test and H. pylori Antibody Test

In 2002, Sipponen *et al* have proposed combining PGI, gastrin-17, and H.pylori antibody using an empirical algorithm to diagnose atrophic gastritis (323, 351). In the initial case-control study in Finland where the algorithm was first proposed, cases were 56 selected dyspeptic outpatients with advanced (moderate or severe) atrophic gastritis and controls were 44 outpatients without advanced atrophic gastritis (323). By using the algorithm, 81% of the patients were classified into the correct "gastritis categories" diagnosed by pathohistology. For discriminating atrophic gastritis in general from non-gastrophic gastritis or normal stomach, this combination had a sensitivity of 89% and specificity of 93%.

Later, the same research group conducted a larger study to validate the usefulness of the algorithm (324). In this study, they enrolled 404 consecutive adult outpatients with various dyspeptic symptoms from five outpatient clinics in Finland. All the patients underwent endoscopy and were tested for serum PGI, gastrin-17 (including fasting gastrin-17 level and gastrin-17 level 20 minutes after protein stimulus), and *H. pylori* antibody. The overall agreement between the algorithm and pathohistology was 81% using fasting gastrin-17 level and 83% using gastrin-17 level 20 minutes after protein stimulus. For diagnosing atrophic gastritis, the sensitivity, specificity, positive predicted value, and negative predicted value were 79%, 91%, 64%, and 93% respectively using fasting gastrin-17 level, and the test using gastrin-17 level 20 minutes after protein stimulus showed slightly better results.

Combining serum biomarkers for diagnosing gastric diseases has gained interests from industry. Sartorius Biohit is a company in Finand that developed the GastroPanel® (a combination of serum PGI, PGII, gastrin-17 and anti-*H. pylori* IgG) as a commercial non-invasive examination to diagnose not only *H. pylori* infection, but also atrophic gastritis and its gastrosubsite location. In addition, they developed the GastroSoft® to interpret the GastroPanel results (http://www.biohit.com/gastropanel-interpretation).

In a study with a relatively big population-based sample (n=976) (352), investigators used a slightly different algorithm from the one proposed by Sipponen *et al.* The overall agreement between this algorithm and pathohistology was 85%. For diagnosing corpus atrophy, the overall agreement was 96%. Sensitivity and specificity of this algorithm for diagnosing atrophic gastritis were 71% and 98%.

Another small study conducted in Spain (353) with 56 patients (47 patients with uninvestigated dyspepsia and 9 consecutive patients with gastric carcinoma) showed that the agreement between the Gastropanel and pathohistology was 68%, and the sensitivity and specificity of the Gastropanel for diagnosing atrophic gastritis were 87.5% and 100%, respectively. However, the Gastropanel failed to detect four of the nine gastric carcinomas because these tumors developed in nonatrophic mucosa.

Another study in Japan enrolled 162 patients who attended outpatient clinic for upper GI endoscopy, and simultaneously performed the GastroPanel examination on these patients (354). Using the endoscopic histology as the gold standard, GastroPanel examination yielded an accuracy of 87%, sensitivity of 40%, and specificity of 95% for diagnosing atrophic gastritis. For discriminating diseased gastric mucosa from healthy

mucosa, GastroPanel examination yielded an accuracy of 94%, sensitivity of 95%, and specificity of 93%.

Another study in the US enrolled 180 randomly selected individuals and failed to confirm the usefulness of the proposed algorithm by using gastrin-17, H pylori serology, and serum pepsinogens to categorize the gastric histology (355). Using the endoscopic histology as the gold standard, GastroPanel examination yielded a sensitivity of 100% and specificity of 12% for diagnosing atrophic gastritis in the group with negative *H. pylori* test; the sensitivity and specificity were 88% and 27% for diagnosing atrophic gastritis in the group with positive *H. pylori* test.

Current Recommendations for Management of Gastric Cancer Precursors

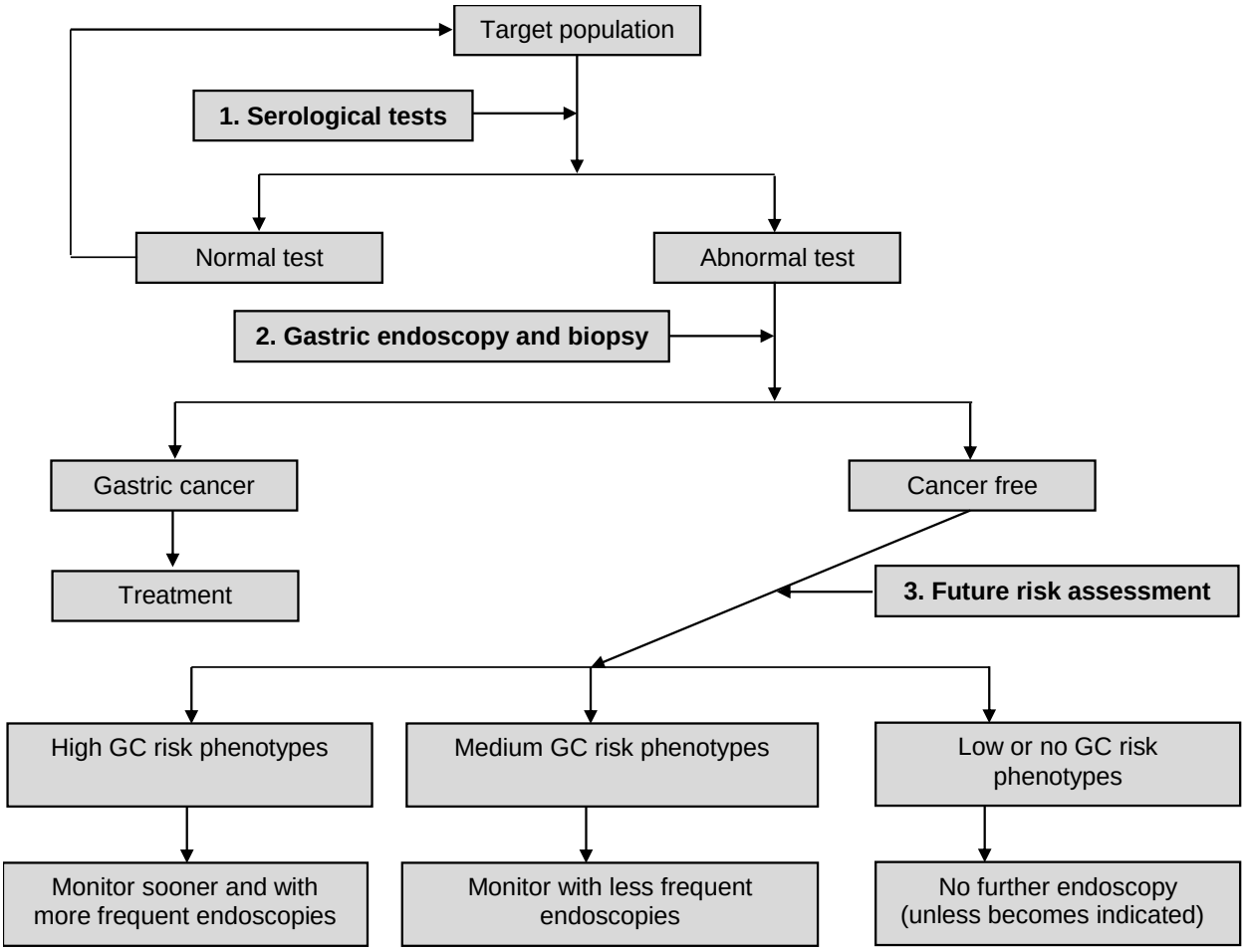
Currently, there is consensus on the management of gastric cancer precursors. According to the recommendations from the European Society of Gastrointestinal Endoscopy (ESGE), European Helicobacter Study Group (EHSG), European Society of Pathology (ESP), and the Sociedade Portuguesa de Endoscopia Digestiva (SPED), endoscopic follow-up should be performed within 0.5 - 3 years depending on the extent of atrophic gastritis/intestinal metaplasia and presence of dysplasia.

A Proposed Screening and Management Strategy for Gastric Cancer and Precursors

Based on current understanding of the progression of gastric precancerous lesions, the molecular basis of gastric carcinogenesis—especially in relation to the causal role of *H. pylori* infection—and the strengths and limitations of current available screening and early detection methods, we propose a new, likely cost-effective screening and early detection strategy that could be used in both developed and developing countries (**Figure 1.2**). There are three basic steps: 1) identification of high-

risk individuals in target populations based on serological findings and clinical history and symptoms; 2) gastric endoscopy with biopsies in the high risk individuals; and 3) differential monitoring according to gastric histology and serum biomarker profile. We propose assessing biomarkers of risk for gastric adenocarcinoma in conjunction with a clinical- or serology-indicated first screening gastric endoscopy to determine whether and when future screening gastric endoscopies are needed. So, for example, if someone had an early precancerous lesion with a low-risk biomarker profile, they may not need screening gastric

Figure 1.2. Proposed screening and early detection strategy for gastric cancer



endoscopy in the near future. On the other hand, if a person with an advanced precancerous lesion had a high-risk biomarker profile, they may need sooner and more frequent future gastric endoscopies, and both the patient and physician may be more motivated to see that this happens.

Morphology of Human Colon and Rectum

The colon and rectum is an important organ of the digestive tract which consists of several regions: cecum, ascending colon, transverse colon, descending colon, sigmoid and rectum. Colon and rectum are lined with a single layer of epithelial cells and most of colorectal cancers arise from the epithelial cells. The single layer of epithelial cells further folded into colorectal crypts. Stem cells are located at the base of the crypts. Stem cells replicate and the daughter cells undergo rapid proliferation and migrate up towards the top. Toward the top of the crypt, cells differentiate and undergo apoptosis. The upper 40% of the crypt is usually as the differentiation zone and the lower 60% as the proliferation zone (356-360).

Descriptive Epidemiology of Colorectal Cancer

New Cases, Deaths: According to the most recent data (361), colorectal cancer is the third most common cancer worldwide with 1,361,000 cases in 2012, and it account for 9.7% of all cancers cases. About 55% of all new cases were from developed countries. colorectal cancer is the fourth leading cause of cancer death worldwide with 694,000 deaths in 2012, and it account for 8.5% of all cancers cases. In the United States colorectal cancer is the fourth most common incident cancer and the second most common cause of cancer deaths (362).

Sex, Age, Race/ethnicity: Unlike GC, which affects men more, colorectal cancer affects men and women approximately equally. In 2012, there were 746,000 and

614,000 new cases in men and women, respectively (361). The incidence rate of colorectal cancer increases progressively with age. In the United States, about 90% of the new cases were diagnosed in people aged 50 years and over (363). colorectal cancer incidence and mortality vary between race/ethnicity groups with the highest rates in African Americans and lowest rates in Asian American/Pacific Islander (363). However, the rates vary greatly even within one race/ethnicity. For example, incidence rates among American Indians/Alaska Natives living in Alaska are 5 times the rates among American Indians/Alaska Natives residing in the Southwest (102.6 *vs.* 21.0 per 100,000) (364).

Geographic Variations and Time Trends: Incidence of colorectal cancer varies about 10- fold worldwide, with the highest rates found in Australia/New Zealand (age-standardized rates: 44.8 and 32.2 per 100,000 in men and women respectively), Europe, and North America, and the lowest rates in Africa (age-standardized rates: 4.5 and 3.8 per 100,000 in men and women respectively in Western Africa) and South-Central Asia (361). There is less variation in mortality worldwide. Immigrants from high- to low-risk areas had a significantly reduced incidence rate (364). Generally, the first generation of immigrants maintains the risk of the homeland, while the risk of subsequent generation approached that of their new country (365). These reports provide compelling evidence that environmental factors can modify the risk for sporadic colorectal cancer. Generally, the incidence has been increasing over the past 35 years worldwide, especially in developing countries such as Slovakia and China. There was a substantial increase of incidence in Japan from 1970s to 1990s and the incidence rate decreased gradually afterwards. The incidence rate has been decreasing substantially in the United States since 1985 (361) probably due to the increase in use of screening sigmoidoscopy or colonoscopy (366).

Sub-sites, Survival: In the United States between 1992 and 2009, about 15%, 11%, 3%, 6%, 3%, 4%, 25%, 27%, 10%, and 23% of CRCs were diagnosed in the caecum, ascending colon, hepatic flexure, transverse colon, splenic flexure, descending colon, sigmoid colon, rectosigmoid junction, and rectum (367). Based on data from SEER 18 2003-2009, the overall 5-year survival was 64.9%. About 40%, 36%, and 20% of CRCs were diagnosed at localized, regional, and distant stage with corresponding 5-year survival rates of 90.3%, 70.4%, and 12.5%.

Risk Factors for Colorectal Cancer

Hereditary Colorectal Cancer: Hereditary conditions such as hereditary non-polyposis colon cancer (HNPCC, or Lynch Syndrome), familial adenomatosis polyposis (FAP), MUTYH-associated polyposis, and certain hamartomatous polyposis conditions account for approximately 5% of all CRCs (368-370). The most common hereditary condition is HNPCC which accounts for 1-4% of all colorectal cancer cases (368-370). Lynch Syndrome is caused by inactivation of the DNA mismatch repair system (primarily *MLH1 and MSH2*) through either germ-line mutation or somatic inactivation of the wild-type allele (371-374). The lifetime risk of colorectal cancer among HNPCC patients is about 80% (370, 375).

In FAP, the affected person is born with an inactivating mutation in one allele of the “pathway gatekeeper” APC tumor suppressor gene (368-370). After an acquired inactivation of the second allele, FAP patients begin to develop hundreds to thousands of adenomas early in life, and the lifetime risk of colorectal cancer among FAP patients is almost 100% if left untreated.

Family History: Among the non-hereditary colorectal cancer, about 30% have a family history of the disease, and about 65% appear to be totally “sporadic”. According

to the most recent meta-analysis which included 43 case-control or cross-sectional studies and 17 prospective or retrospective cohort studies (376), the odds ratio (OR) for individuals with a first-degree relative with colorectal cancer was 2.24 (95% CI 2.06 - 2.43), and the OR was 3.97 (95% CI: 2.60 - 6.06) for individuals with more than one relative with colorectal cancer.

Inflammatory Bowel Disease: Patients with inflammatory bowel disease (i.e., Crohn disease and ulcerative colitis) are increased risk of colorectal cancer compared to the general population. One study found that the risks of colorectal cancer among Crohn's disease patients or ulcerative colitis patients were 2.6 or 2.8 times higher than the risk among the general population, respectively (377).

Diabetes: According to a meta-analysis that included 6 case-control studies and 9 cohort studies (378), the relative risk (RR) for individuals with diabetes was 1.30 (95% CI 1.20 - 1.40) compared with no diabetes, and the associations were similar between the cancers in the colon or rectum.

Smoking: Smoking is significantly associated with increased risk for colorectal cancer. A meta-analysis of 26 studies which provided adjusted risk estimates reported a pooled RR of 1.18 (95% CI 1.11 - 1.25) (379) comparing ever smokers to never smokers. Nicotine, one of the major components of cigarette smoke, can stimulate proliferation of colon cancer cells through epidermal growth factor receptor mediated pathways (380-382).

Aspirin and Other Non-Steroidal Anti-Inflammatory Drugs (NSAIDs): Aspirin and NSAIDs use is one of the factors that have been consistently associated with reduced risk of colorectal cancer (383). Both aspirin and NSAIDs inhibit the COX-1 and COX-2 enzymes, resulting in the inhibition of inflammatory signaling, proliferation, and

angiogenesis, and promotion of apoptosis (384-386). According to the most recent meta-analysis of 12 cohort studies, an increment of 325 mg aspirin per day was associated with a 20% statistically significant decreased risk of colorectal cancer, 7 times per week increment with 18% decreased risk and 10 years of use increment with 18% decreased risk (387). All studies that investigated non-aspirin NSAIDs in relation to colorectal cancer risk found that non-aspirin NSAIDs use was consistently associated with reduced risk of colorectal cancer (388-390).

In a pooled analysis of four randomized trials of lower dose of aspirin (75-300 mg daily) versus control for primary or secondary prevention of vascular events over 20 years (391), aspirin use reduced the risk of colon cancer incidence and mortality by 24% and 35%, respectively, but there was no significant effect on rectal cancer. The benefit increased with duration of aspirin treatment (i.e., aspirin use for 5 or more years reduced overall incidence by 32%, incidence of proximal colon cancer by 65%, and incidence of rectal cancer by 42% (391)). However in the Women's Health Study (n = 39, 876, age ≥ 45 years), aspirin (100 mg) every other day compared to placebo for an average of 10 years had no effect on the incidence of any cancer (392). Also, in the Physicians' Health Study (n = 22,071, males), aspirin (325 mg) every other day compared to placebo had no effect on the incidence of colorectal cancer after 5 years of follow-up (393) and at 12 years follow-up (394). Another randomized clinical trial involving 1071 carriers of Lynch syndrome of 600mg aspirin use for 4 years did not support the protective effect of aspirin (395).

In a pooled analysis of four randomized trials of lower dose of aspirin (81-325 mg daily) versus control for secondary prevention of colorectal adenomas over 33 months (391), aspirin use reduced the risk of any recurrent adenoma by 17% (RR = 0.83, 95%

CI = 0.72 to 0.96) and risk of advanced lesion by 28% (RR = 0.72, 95% CI = 0.57 to 0.90).

Three randomized controlled trials reported that selective COX-2 inhibitors (celecoxib 200-400 mg/day or rofecoxib 25 mg/day) reduced the risk of recurrent colorectal adenomas by 24 - 45% over 3 years follow-up (396-398); however, all three studies also reported associated with increased risks of significant upper gastrointestinal events and serious cardiovascular events in the active treatment groups (396-398).

Taken together, current evidence shows that aspirin and NSAIDs, taken in doses higher than those recommended for prevention of cardiovascular disease and long duration (e.g., > 5 years), reduces the incidence of colorectal adenomas and cancer. However, aspirin and NSAIDs use is associated with increased risk of gastrointestinal bleeding and cardiovascular events. The U.S. Preventive Services Task Force concludes that harms outweigh the benefits of aspirin and NSAID use for the prevention of colorectal cancer (399).

***Physical Activity, Body Composition, Drinking, Food, and Nutrition*:** The World Cancer Research Fund (WCRF) rigorously reviewed the available evidence on the associations of physical activity, body composition, drinking, food, and nutrition with colorectal cancer risk. The 2007 World Cancer Research Fund report concludes that there is convincing evidence that lack of physical activity, fatness, higher adult attained height, read/processed meat and alcoholic drinks (men) were associated with increased risk of colorectal cancer (400).

Two recent meta-analyses estimated an approximately 20% lower risk for colon cancer when comparing the most vs. least active individuals; however the protective effect did not seem to apply to rectal cancer (401, 402). However most studies might not

be suitable for meta-analysis due to the disparate measures used to assess physical activity (400). According to a meta-analysis which included thirty prospective studies (403), a 5-unit increase in body mass index (BMI; in kg/m(2)) was associated with an 30% increased risk of colon cancer in men and 12% increased risk in women; a 5-unit increase in BMI was only associated with an increased risk of rectal cancer in men (RR: 1.12; 95% CI: 1.09, 1.16)) but not in women (RR: 1.03; 95% CI: 0.99, 1.08). The 2007 World Cancer Research Fund report showed that a 5% increased risk was associated with one inch of waist circumference, and a 30% increased risk with 0.1 increment of waist to hip ratio (400). According to meta-analysis of cohort data, a 9% increased risk was associated with 5 cm of height (400).

Meta-analysis of 16 cohort studies showed a 43% increased risk per time consumed/week of red meat intake or a 15% increased risk per 50 g/day of red meat intake (400). Meta-analysis of 14 cohort studies showed a 21% increased risk per 50 g/day of processed meat intake (400). Meta-analysis of 24 cohort studies showed a 9% increased risk per 10 g ethanol/day, and the adverse effect seemed to be stronger among men than among women (400).

Calcium and Vitamin D

The 2007 World Cancer Research Fund report concludes that higher calcium and vitamin D intake are associated with reduced risk of colorectal cancer with probable and limited-suggestive evidence, respectively (400).

The analytic observational literature evidence is intensive and supportive regarding whether calcium reduces risk for colorectal cancer in humans. Of at least 46 analytic epidemiologic studies (404-449) (24 case-control studies (404-423, 447, 448) and 22 prospective cohorts (424-446, 449)), 37 reported an inverse association between

calcium intake and colorectal neoplasms (405, 406, 408, 410-413, 415, 417, 419-426, 428-434, 436-440, 442-449), one reported non-statistically significant increases risk (435), and the other 8 reported no association. According to a recent meta-analysis of 17 cohort studies reported a summary RR of 0.77 comparing highest to lowest intake (450).

To date, there have been four randomized, placebo-controlled, clinical trials that tested the effects of calcium against adenoma recurrence (451-453) and colorectal cancer risk (454), and one reported statistically significant protective effect (1200 mg/day, RR = 0.83, 95% CI = 0.68 - 1.00) (451), two (452, 453) reported statistically significant protective effect (1600 mg/day, RR = 0.79, 95% CI = 0.50 - 1.23; 2000 mg/day, RR = 0.71, 95% CI = 0.50 - 1.01), and one (454) found no effect (1000 mg/day, HR = 1.08, 95% CI = 0.86 - 1.34). A subsequent analysis (455) found that the effect of calcium on colorectal adenoma recurrence was modified by baseline 25(OH)D concentrations, and the protective effect was only seen among the individuals with baseline 25(OH)D concentrations above 29.1 ng/ml (RR = 0.71; 95% CI: 0.57 – 0.89 vs. RR = 1.05; 95% CI: 0.85 – 1.29).

The three most prominent mechanisms of calcium against colorectal cancer include protection of the colorectal mucosa against bile acids, direct effects on the cell cycle, and modulation of E-cadherin and β-catenin expression in the APC colon carcinogenesis pathway (456, 457).

There are two main sources of vitamin D: 1) synthesis in the human skin under UV light and 2) dietary intake of vitamin D_3. Circulating vitamin D_3 is metabolized by the CYP27A1 enzyme in the liver to form 25-hydroxyvitamin D ($25(OH)D_3$) and by the CYP27B1 enzyme in the kidney to form $1,25(OH)_2D_3$ (457). $25(OH)D_3$ is the primary

circulating form of vitamin D and regarded as a useful biomarkers of vitamin D exposure integrating dietary intake, supplements and exposure to ultraviolet light (458), and $1,25(OH)_2D_3$ is the active form of vitamin D.

A total of 16 prospective cohorts have investigated the association between vitamin D and colorectal neoplasms (424, 428, 432, 436, 449, 459-469), with 13 suggesting an inverse association (424, 428, 432, 436, 449, 460-464, 466, 468, 469). A recent meta-analysis of vitamin D intake (nine studies) and blood 25(OH)D levels (nine studies) with colorectal cancer risk reported a summary RR of 0.88 (95% CI, 0.80 - 0.96) comparing highest to lowest categories of vitamin D intake and a RR of 0.74 (95% CI, 0.63 - 0.89) for a 10 ng/ml increment of blood 25(OH)D levels. To date, there is only one randomized, placebo-controlled, clinical trial that tested the effect of vitamin D supplement against colorectal cancer (454). Specifically, a daily dose of 400 IU of vitamin D for an average of 7 years did not reduce the risk of colorectal cancer among 36,282 postmenopausal women.

The four most prominent mechanisms for vitamin D include bile-acid catabolism, direct effects on the cell cycle, growth-factor signaling, and immunomodulation (456, 457).

Molecular Basis of Colorectal Cancer

Colorectal carcinogenesis is one of the most studied carcinogenic processes and one of the classical examples of a multistage carcinogenesis (6, 371). With the accumulation of genetic/epigenetic alterations and imbalance of growth factors that promote uncontrolled growth, replication, and evasion of apoptosis, normal colorectal mucosa progresses to adenoma and finally to colorectal cancer (6, 371).

There are three major pathways that drive colorectal neoplasias: chromosomal instability (CIN), microsatellite instability (MSI), CpG island methylator phenotype (CIMP) (371, 470). Approximately 12-15% of all CRCs showed MSI which is caused by inactivation of the DNA mismatch repair system, and most colorectal cancers acquire genomic instability by CIN or CIMP (470).

CIN, which causes changes in chromosomal copy number and structure (471), is the most common type of genetic instability in CRCs (371). The chromosomes most frequently deleted include 5q, 17p, and 18q (6). CIN can cause the physical loss of a wild-type copy of a tumor-suppressor gene, such as *APC*, *P53*, and SMAD family member 4 (*SMAD4*) (371). MSI is caused by inactivation of the DNA mismatch repair system (primarily *MLH1* and *MSH2*) either through germ-line mutation or somatic inactivation due to promoter methylation (primarily *MLH1*) (371-374). Deficiency in DNA mismatch repair system leads to inactivation of tumor-suppressor genes, such as those encoding transforming growth factor β (*TGF-β*) receptor type II (*TGFBR2*) and BCL2-associated X protein (*BAX*) (371). Aberrant methylation in promoter regions can induce epigenetic silencing of gene expression (472), and CIMP is a phenomenon when a subgroup of the loci that can undergo aberrant methlation tends to become aberrantly methylated as a group (472). CIMP is present in about 15% of CRCs and in almost all CRCs with promoter methylation primarily *MLH1* (472-475).

It now appears that there are two major pathways by which colorectal cancer develops: the “APC Pathway” and the “Mismatch Repair (MMR) Pathway”. The “APC Pathway” accounts for FAP and approximately 80% of sporadic CRCs. In FAP, the affected person inherits an inactivating mutation in one allele of the “pathway gatekeeper” APC tumor suppressor gene and acquires inactivation of the second allele (368-370). In the sporadic colorectal cancer patients, inactivation of both alleles must be acquired,

either through somatic mutation or epigenetic phenomena, predominantly the former. APC degrades β-catenin which is both pro-proliferative and regulates E-cadherin, a calcium-dependent cell adhesion molecule necessary for colon crypt structure and function. Increased cell proliferation and altered cell adhesion are hallmarks of the progression from normal colorectal epithelium to adenoma to carcinoma.

The "Mismatch Repair (MMR) Pathway" accounts for HNPCC and approximately 20% of sporadic CRCs. HNPCC patients inherit an inactivation of genes of the DNA mismatch repair system (primarily *MLH1 and MSH2*) (371-374). Sporadic colorectal cancer patients in this pathway must acquire an inactivation through genetic mutation or inactivation due to promoter methylation (primarily *MLH1*) (371-374). Deficiency in DNA mismatch repair system leads to inactivation of tumor-suppressor genes, such as those encoding transforming growth factor β (*TGF-β*) receptor type II (*TGFBR2*) and BCL2-associated X protein (*BAX*) (371). The net effect is increased cell proliferation and decreased cell apoptosis and differentiation.

TGFα and TGFβ_1

Transforming growth factor alpha (TGFα) and transforming growth factor beta 1 (TGFβ_1) are autocrine/paracrine growth factors that are classically thought of as potent promoters and inhibitors of cell growth, respectively, in normal tissues (476, 477), and likely contribute to or at least affect colorectal carcinogenesis (478). TGFα acts through the EGF receptor, and it was previously reported that, in the normal colorectal mucosa, immunohistochemically-detected TGFα was denser in the upper one-third to two thirds of colonic crypts (479-481) (differentiation zone).

TGFβ has three isoforms, including TGFβ_1, TGFβ_2, and TGFβ_3; TGFβ_1 is expressed in epithelial cells (482). The growth inhibitory signal of TGFβ is transduced

through two receptors, type I (RI) and type II (RII) (482), and both receptors are abundantly expressed in the normal colon epithelium (483). TGFβRII inactivation is found in colorectal cancers and related cell lines with microsatellite instability (MSI) (484, 485), and MSI colorectal tumors tend to arise in the proximal colon (485). $TGF\beta_1$ immunoreactivity was previously reported to localize mainly in the upper third of the crypts of the normal colorectal mucosa (483). $TGF\beta_1$ signaling, which is complex in cancer progression, regulates cell proliferation, apoptosis, autophagy, inflammation, tumor angiogenesis, and metastasis (482). A dual role of $TGF\beta_1$ has been proposed, because $TGF\beta_1$ suppresses the growth of normal epithelial cells but promotes tumor metastasis in later stages of cancer (482). In a mouse model, the absence of $TGF\beta_1$ expression promoted the progression from hyperplasia to adenoma and allowed the development of carcinoma (486).

The Colorectal Adenoma - Carcinoma Sequence

There are several types of colorectal polyps, including hyperplastic polyps, inflammatory polyps, non-neoplastic hamartomas (juvenile polyps), lymphoid, dysplasia-associated lesion or mass (DALM) , and adenomatous polyps (adenomas) (487). Colorectal adenoma is widely accepted as the precursor for colorectal cancer (6, 371, 488, 489) and about 95% of sporadic colorectal cancers develop from adenomas (490). It is estimated that about 5 to 10 years are required for the progression of adenomas to malignancy (7, 8, 491). Only less than 1% of all adenomas develop into colorectal cancers (492), and the likelihood of progression of adenomas into colorectal cancers depends on the size, multiplicity, histologic features, and appearance of the adenomas (492). Adenomas can be divided into tubular, villous, or tubulovillous adenomas according to histologic features, or into pedunculated/stalked, or sessile/flat-based adenomas according to appearance (492). High-risk adenomas refer to tubular

adenoma ≥ 10 mm, 3 or more adenomas, adenoma with villous histology, or high grade dysplasia (10).

The colorectal adenoma - carcinoma sequence has been supported by epidemiological, clinicopathogical, genetic, molecular genetic, cytogenetic, molecular cytogenetic, cytometric, gene expression evidence (6, 371, 488, 493). According to a recent meta-analysis (n=18), the prevalence of adenomas in average-risk North Americans was 30.2% (range, 22.2% - 58.2%) using traditional colonoscopy (494). The prevalence could be higher if modern technology (such as high-definition white light) were used. However, recent evidence suggested that hyperplastic polyps can give rise to serrated polyps which may progress to colorectal cancers (495).

Current Recommendations for Management of Colorectal Adenoma

Back in the 1970s, adenoma patients were endoscopically examined annually to detect new and missed adenomas (496). In 1993, the landmark National Polyp Study (497) showed clearly that the first post polypectomy endoscopic follow-up could be deferred for 3 years. In 2003, it was recommended that adenoma patients should be stratified into low risk and higher risk for subsequent adenomas accord to baseline adenoma characteristics (491). According to the current recommendations of the US Multi-Society Task Force on Colorectal Cancer (10), endoscopic follow-up should be performed within 3 - 10 years depending on the size, multiplicity, histological type, and presence of dysplasia.

Gaps in the Literature Addressed by This Dissertation

As extensively reviewed above, gastric cancer and colorectal cancer remain huge burdens worldwide, and the numbers of new cases are expected to increase with expanding populations and increasing colorectal cancer risk in developing countries.

The precursors of gastric cancer and colorectal cancer are well established, and it follows that effective management of precursors could lead to reduced gastric cancer and colorectal cancer incidence and mortality. Currently, there is no broad, interrelated consensus on identification and response to precursors of gastric cancer and colorectal cancer. It has been stated that active surveillance is required for patients with precancerous lesions. Currently, management of gastric cancer and colorectal cancer precursors is only based on histopathological findings, and biomarkers are not yet used to improve management.

For patients with gastric cancer and colorectal cancer precursors, no chemopreventive agents have been recommended with certainty. However, chemoprevention trials with gastric cancer or colorectal cancer incidence or mortality as the endpoints are limited due to the extended time to develop these cancers and the large sample sizes and high costs involved. Therefore, modifiable pre-neoplastic biomarkers of risk for gastric and colorectal neoplasms that could be used as surrogate endpoints to investigate the potential efficacy of preventive interventions in short-term clinical trials are needed to assess potential efficacy, optimal dose, and safety.

In order to address these gaps in the literature, I evaluated potential roles for plausible gastric cancer- and colorectal cancer-related biomarkers for 1) identifying precursor lesions, 2) risk stratification, and 3) surrogate endpoints in chemoprevention trials to assess the potential efficacy, safety, and optimal dose of interventions.

Chapter 2. Objectives, Specific Aims, and Hypotheses

Overall Goal

The overall goal of this dissertation is to evaluate potential roles for plausible GC- and CRC-related biomarkers for 1) identifying precursor lesions, 2) risk stratification, and 3) surrogate endpoints in chemoprevention trials to assess the potential efficacy, safety, and optimal dose of interventions.

Specific Aims

1. Investigate whether serum levels of pepsinogen I (PGI), PGII, PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG, individually or combined, are sufficiently accurate to be used as biomarkers for identifying abnormal gastric histopathologies, including gastric cancer and its precursors.

2. Investigate whether temporal changes in PGI, PGII, PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG are associated with risk for progression of gastric precancerous lesions.

3. Investigate whether the expression of TGFα and/or $TGF\beta_1$ (autocrine/paracrine growth-promoting and -inhibiting factors, respectively) in biopsies of normal-appearing colorectal mucosa differs by incident sporadic colorectal adenoma case-control status (i.e., could be valid biomarkers of risk for colorectal neoplasms).

4. Estimate the effects of supplemental calcium 2,000 mg (as calcium carbonate given in two equal divided doses daily with food) and vitamin D_3 800 IU daily over 6 months on the expression of TGFα and TGFβ_1 in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients.

The first two research questions will be addressed by using data from the Zhuanghe Gastric Diseases Screening Program (n = 10,635), a population-based, combined serologic/endoscopic screening program for gastric diseases, particularly GC, in Zhuanghe County in northern China since 1997 in which repeated gastroscopies with gastric mucosal biopsies and blood sample collections were conducted on 2,039 participants (5,070 person-visits). The third research question will be addressed by using data from a pilot colonoscopy-based case-control study (49 cases / 154 controls), and the fourth research question will be addressed by using data from a pilot, randomized, double-blind, placebo-controlled, 2 x 2 factorial clinical trial (n = 92) of calcium 2,000 mg and/or vitamin D_3 800 IU daily over 6 months.

Hypotheses

1. I hypothesize that the distributions of the five biomarkers (i.e., serum PGI, PGII, PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG) are different across different histopathological conditions (i.e., normal, non-atrophic gastritis, atrophic gastritis, and neoplastic lesions, including dysplasia, polyps, and gastric adenocarcinoma). I also hypothesize that each of the five biomarkers alone has insufficient sensitivity and specificity for screening for gastric diseases, including gastric cancer and its precursors, and that combining the five biomarkers will increase the accuracy of screening for gastric diseases.

2. I hypothesize that temporal changes in the serum biomarker profile predict the progression of gastric precancerous lesions at follow-up visits. I also hypothesize that combining the five biomarkers will increase the prediction ability for progression of gastric precancerous lesions.

3. I hypothesize that the expression of TGFα and TGFα/TGFβ_1 ratio will be greater in biopsies of normal-appearing colorectal mucosa of incident sporadic colorectal adenoma patients than in adenoma-free patients. Also, I also hypothesize that TGFβ_1 expression will be less in biopsies of normal-appearing colorectal mucosa of sporadic colorectal adenoma patients than in adenoma-free patients.

4. I hypothesize that supplemental calcium and vitamin D_3 can decrease TGFα expression and the TGFα/TGFβ_1 expression ratio and increase TGFβ_1 expression in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients.

Chapter 3. Serological Biomarkers for Identification of Precancerous Gastric Lesions

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Abbreviations:

AG: atrophic gastritis

ELISA: enzyme-linked immunosorbent assay

GC: gastric cancer

GS: superficial gastritis

H. pylori: Helicobacter pylori

IgG: immunoglobulin G

NAG: non-atrophic gastritis

PG: pepsinogen

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Author Contributions: YY conceived and designed this study and revised the manuscript. HT, XD, QL performed data interpretation and statistical analysis and wrote the paper. YY and LS were responsible for collection of the serum/biopsy samples used for the analysis and histopathological examinations. YG, QX, and JJ were responsible for serological testing. RMB participated in data analysis and interpretation and manuscript writing. All authors read and approved the final version of the report.

Key words: Non-invasive tests; gastric precancerous lesions; cost-effectiveness; gastric cancer

Abstract

Background and Aims: We evaluated using multiple serological biomarkers (pepsinogen I [PGI], PGII, PGI/II ratio, anti-*H. pylori* IgG and gastrin-17), individually and combined, to identify high gastric cancer (GC) risk individuals for assessment/management of future gastric cancer risk.

Methods: Data were from a population-based endoscopic, gastric diseases screening program in northern China. From 1997 to 2011, gastroscopies with mucosal biopsies were conducted on 10,635 participants. Serum biomarkers were measured using enzyme-linked immunosorbent assays (ELISA), and gastric biopsies were evaluated using standardized criteria. Logistic regression was used to produce receiver operator characteristic (ROC) curves with corresponding c statistics.

Results: For identifying the presence of any abnormal gastric condition (including moderate/severe non-atrophic gastritis, atrophic gastritis, dysplasia, and GC), PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG, and gastrin-17 individually yielded c statistics of 0.57, 0.71, 0.70, 0.74, and 0.58, respectively. PGII and anti-*H. pylori* IgG combined provided the same c statistic as for the five biomarkers combined (c = 0.77). A combined test of PGII ≥ 8.25 ng/ml plus anti-*H. pylori* IgG ≥ 24.02 EIU yielded a sensitivity of 80.7% and specificity of 52.8%. For screening for gastric cancer specifically, PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG, and gastrin-17 individually yielded c statistics of 0.53, 0.55, 0.61, 0.52, and 0.51, respectively, and the five biomarkers combined yielded a c statistic of 0.61.

Conclusions: Our results suggest that serum PGII combined with anti-*H. pylori* IgG may be useful for aiding screening for abnormal gastric conditions and the need for assessment/management of future gastric cancer risk.

Introduction

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer deaths worldwide, with 989,600 incident cases and 738,000 deaths in 2008 (1). About 72% of incident cases occur in developing countries (1), with 42% in China alone (24). It is well accepted that GC, especially the intestinal type, is preceded by a prolonged precancerous process [Correa's model(2-5)] in which gastric mucosa progresses from “normal gastric mucosa → non-atrophic gastritis → atrophic gastritis → intestinal metaplasia → dysplasia → invasive adenocarcinoma”. Therefore, effective management of precancerous gastric lesions could lead to reduced gastric cancer incidence and mortality.

Currently, there is no consensus on how to manage patients with gastric precancerous lesions. We propose a new, likely cost-effective management strategy that could be used in both developed and developing countries. There are three basic steps: 1) identification of individuals with precancerous lesions in target populations based on serological findings, clinical history, and symptoms; 2) gastric endoscopy with tissue biomarker testing in the high risk individuals; and 3) differential monitoring according to gastric histology and molecular phenotypes.

In this strategy, we first need to identify those individuals with gastric precancerous lesions. Gastroscopy (with biopsies for histological examination) is commonly accepted as the “gold standard” for diagnosing precancerous lesions and detecting gastric cancer (498, 499). However, gastroscopy use is limited by its invasiveness and an insufficient supply of skilled endoscopists and endoscopy facilities, even in highly developed countries such as Japan (499). Serological tests are less invasive, more accessible, less expensive, and less time-consuming than gastroscopy. It has been proposed that serological tests for pepsinogens (PGs), anti-*Helicobacter pylori*

(*H. pylori*) antibody, and gastrin-17 might be useful for identifying high gastric cancer risk individuals (*e.g.*, those with gastric precancerous lesions) who should be referred for gastroscopy (9, 499).

To date, no studies have reported on the accuracy of PGs, *H. pylori* antibody, or gastrin-17 tests individually or combined for identifying gastric precancerous lesions. In the present study, we investigated the accuracy of five serological biomarkers (PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG, and gastrin-17), individually and combined, for identifying abnormal gastric conditions (including gastric precancerous lesions and GC) in a large cohort of participants in a gastric diseases screening program in Zhuanghe County in northern China.

Material and Methods

Study population

The Zhuanghe Gastric Diseases Screening Program is a population-based, combined serologic/endoscopic screening program for gastric diseases, particularly GC, that has been conducted in Zhuanghe County, a high gastric cancer risk area in China, since 1997. The study population selection and recruitment process is summarized in **Figure3.1**.

A sample of 50 villages with previously reported gastric cancer cases was selected to geographically represent all the villages in Zhuanghe County. The program targets all residents in these 50 villages who are 35 to 70 years old or who have gastrointestinal symptoms (including abdominal bloating, heartburn, acid reflux, nausea, hiccups, belching, decreased appetite, and stomachache) or a positive family history of GC. Participation was voluntary, and to date, 18,760 participants have been recruited.

After excluding those without a gastric histopathological diagnosis (n = 7,833) or biomarker measurements (n = 234), and those with a history of a gastrectomy (n = 58), 10,635 participants, including 110 newly diagnosed gastric cancer cases and 10,525 non-cases, were included in the final analysis.

This study was approved by the Human Ethics Review Committee of the First Affiliated Hospital of China Medical University (Shenyang, China). Written informed consent was obtained from each participant in accordance with the Declaration of Helsinki and its later revision.

Serological measurements

A 5 ml fasting venous blood sample was collected from each participant. All samples were centrifuged immediately at 3,500×g for 10 minutes, and serum was immediately frozen and stored until analysis. Serum PGI, PGII, anti-*H. pylori* IgG, and gastrin-17 were measured using enzyme-linked immunosorbent assays (ELISAs) (Pepsinogen I ELISA; Pepsinogen II ELISA; *Helicobacter pylori* IgG ELISA; and Gastrin-17 ELISA kit, BIOHIT Plc, Helsinki, Finland) according to the manufacturer's protocols, blinded to the histopathological diagnosis. Samples that yielded implausible values were re-tested. Duplicate negative and positive controls were included in each 96-well plate. The mean intra-assay coefficients of variation (CV) were 11% for PGI, 12% for PGII, 11% for anti-*H. pylori* IgG, and 15% for gastrin-17.

Endoscopic and histopathological examinations

Experienced endoscopists blinded to the patients' serological results performed the gastrointestinal endoscopies. Mucosal biopsies were obtained from the gastric body, angulus, antrum, and, if applicable, lesion site. The biopsies were oriented, fixed in 95% ethanol, embedded in paraffin blocks, and then sectioned and stained with hematoxylin

and eosin in local study centers. Each stained section was independently evaluated by two gastrointestinal pathologists using standard criteria from the WHO classification for gastric cancer (500) and the visual analog scale of the updated Sydney System for gastritis (501). For histologic sections on which there was initial disagreement on the histopathologic interpretation, the final results were determined through adjudication among the two pathologists and a third pathologist. Each participant was assigned a global diagnosis based on the most severe lesion found among all the biopsy specimens.

Accordingly, the 10,635 included subjects with a histopathologic diagnosis were classified as: normal mucosa/mild non-atrophic gastritis (n = 2,287), moderate/severe non-atrophic gastritis (n = 4,450), atrophic gastritis/intestinal metaplasia (n = 3,294), dysplasia (n = 444), gastric cancer (n = 110), and unclassified (n = 50).

Statistical analyses

All statistical analyses were performed using SAS 9.3 statistical software (SAS Institute Inc., Cary, NC, USA). A P value ≤ 0.05 (two-sided) was considered statistically significant. Odds ratios (OR) with 95 percent confidence intervals (95% CI) were calculated as measures of association using logistic regression (because the goal of the study was the potential predictive ability of the biomarkers, not etiology, the estimates were not adjusted for covariates). Receiver operator characteristic (ROC) curves with corresponding c statistics (area under the curve, AUC) based on logistic models were used to measure the discriminatory performance of each predictor or combination of predictors where the pathologic diagnosis was treated as the "gold standard".

Results

Selected characteristics of the study population

Selected characteristics of the study participants by gastric condition are summarized in **Table 3.1**. Participants with abnormal gastric conditions were more likely to be men, and, on average, to be older and have higher levels of serum PGI, PGII, anti-*H. pylori* IgG, and gastrin-17 and a lower serum PGI/II ratio. Among participants with abnormal gastric conditions, most had either moderate/severe non-atrophic gastritis (53.3%) or atrophic gastritis/intestinal metaplasia (39.5%). A total of 52.3% of the gastric cancer cases had intestinal type gastric adenocarcinomas, and 40.8% had GCs in the stomach antrum.

Serum biomarker concentrations across gastric histopathologic conditions

As shown in **Table 3.2**, from normal mucosa/mild non-atrophic gastritis to moderate/severe non-atrophic gastritis, PGI, PGII, anti-*H. pylori* IgG, and gastrin-17 increased and the PGI/II ratio decreased substantially. Across the histopathologic conditions from moderate/severe non-atrophic gastritis, atrophic gastritis/intestinal metaplasia, dysplasia, to GC, there were no substantial differences or trends in the biomarker levels.

Associations of serum biomarkers with gastric histopathologic conditions

Crude associations of the serum biomarkers with gastric histopathologic conditions, including GC, are presented in **Table 3.3**. Those in the highest quartile of PGI or gastrin-17 relative to those in the lowest quartile had statistically significant 86% or 97% higher odds of having abnormal gastric conditions. For the association of PGII with abnormal gastric conditions, the odds ratios for those in the second, third, and fourth quartiles relative to those in the lowest, were, respectively, 1.26, 3.13, and 8.06, all of which were statistically significant. Those in the lowest quartile of the PGI/II ratio relative to those in the highest had statistically significant nearly 7.3-fold higher odds of

having an abnormal gastric condition. For the association of anti-*H. pylori* IgG titers with abnormal gastric conditions, the odds ratios for those in the second, third, and fourth quartiles relative to those in the lowest, were, respectively, 2.06, 4.54, and 14.10, all of which were statistically significant.

Those in the highest quartile of PGII relative to those in the lowest quartile had statistically significant 85% higher odds of having GC. Those in the lowest quartile of the PG/I/II ratio relative to those in the highest had statistically significant nearly 2.5-fold higher odds of having GC. For the association of anti-*H. pylori* IgG titers with GC, the odds ratios for those in the second, third, and fourth quartiles relative to those in the lowest, were, respectively, 2.13, 1.99, and 1.67, all of which except the latter were statistically significant.

Accuracy of serum biomarkers for screening for gastric histopathologic conditions

Figure 3.2 panel A shows the ROC analysis to assess the screening accuracy of serum PGI, PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG and gastrin-17, individually and combined, for discriminating between those with and without an abnormal gastric mucosa. Of the five included biomarkers, anti-*H. pylori* IgG yielded the highest c statistic (c = 0.74). PGII and anti-*H. pylori* IgG combined yielded the same c statistic (c = 0.77) as that yielded by all five biomarkers combined. According to cut-off points suggested as optimal by our previous studies of PGII (502) and anti-*H. pylori* IgG (342), a combined test of PGII ≥ 8.3 ng/ml plus anti-*H. pylori* IgG ≥ 24.0 EIU yielded a sensitivity of 80.7% and specificity of 52.8%.

Figure 3.2 panel B shows the ROC analysis of serum PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG and gastrin-17, individually and combined, for discriminating between

those with and without GC. For gastric cancer of any histologic type, the five markers, individually or combined, all yielded c statistics ≤ 0.61, with the largest c statistic of 0.61 for the PGI/II ratio individually as well as for the five markers combined. When stratified on different gastric cancer histological types (**Figure 3.3)**, the discriminatory performances tended to be slightly better for the intestinal type than for the diffuse type of gastric adenocarcinoma (e.g., for all biomarkers combined, the c statistics were, respectively, 0.68 and 0.59).

Discussion

Our results suggest that in a rural population in China with high rates of gastric cancer (GC), serum PGII and anti-*H. pylori* IgG, especially combined, may provide adequate accuracy for aiding clinicians or large screening programs in identifying persons with abnormal gastric conditions that may require more invasive or intensive risk assessment or monitoring. Our results also suggest that PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG and gastrin-17, individually or otherwise combined, yield insufficient accuracy for screening specifically for GC.

There are two isoforms of PG: PGI and PGII (290). PGI is only produced in the gastric fundic glandular mucosa, whereas the distribution of PGII-producing cells includes the entire stomach and duodenum (294). During early stage gastric carcinogenesis, serum levels of both PGI and PGII increase due to *H. pylori*-induced inflammation stimulation, but PGII increases to a greater extent, resulting in a lower PGI/II ratio (503); during late stages of gastric carcinogenesis, as chronic atrophic gastritis progresses, mucosal atrophy spreads from the pyloric glands up to the fundic glands, where the PGI secreting cells reside, resulting in decreased PGI, relatively constant PGII, and a further decrease in the PGI/PGII ratio (296).

We found that serum PGII was substantially and similarly elevated during early and late gastric carcinogenesis stages. We also found that PGII yielded acceptable screening accuracy for aiding in identifying abnormal gastric conditions but poor accuracy for screening specifically for GC. Serum PGII has long been neglected in clinical and population-based screening practice (502), but our results suggest that PGII is a potential risk assessment/prescreening test to help identify high gastric cancer risk individuals who may require more invasive (i.e., gastroscopy) or intensive risk assessment or monitoring.

It is commonly accepted that low PGI and/or a low PGI/II ratio in the serum indicate chronic atrophic gastritis, a precancerous lesion for gastric cancer (296), and a combined test of PGI and PGI/II ratio (e.g., PGI ≤ 70 ng/ml plus a PGI/PGII ratio ≤ 3.0) has been used to screen for GC, primarily in Japan and a few other countries (296, 299, 504). Multiple population-based studies in Japan evaluated the accuracy of the combined PG test for gastric cancer screening (505-508), and the sensitivity ranged from 36.8% to 84.6% and the specificity from 65% to 96.0%. In our study, the sensitivity and specificity of the combined PG test were 33.6% and 80.8% (data not shown). As noted by others (332, 499), the combined PG test mainly indicates atrophic lesions, so it is more applicable to the intestinal type of GC. In our study population, the diffuse type of gastric cancer accounted for about half of all gastric cancer cases, so it is unsurprising that the combined PG test did not perform well.

H. pylori seropositivity has been consistently associated with incident gastric cancer risk (162). Nevertheless, to our knowledge, no population-based study has reported on the accuracy of serum anti-*H. pylori* IgG titers for gastric cancer screening or identification of high risk individuals. We found that anti-*H. pylori* IgG individually yielded poor screening accuracy for gastric cancer but acceptable accuracy for aiding in

detecting abnormal gastric conditions combined, making serum anti-*H. pylori* IgG another potential risk assessment/prescreening test to identify high GC-risk individuals who may require more invasive (i.e., gastroscopy) or intensive risk assessment or monitoring.

Japanese researchers developed the so-called “ABC(D)” method, which combines PG and anti-*H. pylori* IgG to predict incident gastric cancer risk (509). In the “ABC(D)” method, based on serum anti-*H. pylori* IgG titer and pepsinogens (i.e., PGI + the PGI/II ratio), individuals are classified into the following four groups: group A [HP(−)PG(−)], group B [HP(+)PG(−)], group C [HP(+)PG(+)], and group D [HP(−)PG(+)]; group A is excluded from subsequent endoscopic examination (509). Consistent with the results from previous prospective studies in which the highest gastric cancer risk was observed among group D (305, 509) or group C (312, 314) individuals, we found gastric cancer prevalence to be highest among group D individuals and second highest among group C individuals (data not shown). However, if group A was excluded from endoscopic examination in our study, we would have missed 40% of all gastric cancer cases (data not shown).

Gastrin-17, released by the antral G cells, has been proposed as a marker of atrophy in the antrum (323) and of hypo- or achlorhydria induced by atrophic gastritis (510), thus suggesting serum gastrin-17 as a potential screening test for gastric cancer (499). To date, no population-based study has reported on the accuracy of gastrin-17 for gastric cancer screening and identification of high risk individuals. We found that high gastrin-17 levels were not associated with gastric cancer risk but that they were modestly associated with all abnormal gastric conditions combined. More studies with better measurement of gastrin-17 (e.g., protein stimulated gastrin-17 level) are needed to evaluate a possible role for gastrin-17 in gastric cancer screening (332).

A research group in Finland developed the “Gastropanel” method, which combines serum PGs, anti-*H. pylori* IgG titers, and gastrin-17 to classify site-specific precancerous lesions (351). In the “Gastropanel” method, GC-free individuals are classified into the following different groups using a decision tree algorithm: normal, non-atrophic gastritis, atrophic gastritis in the corpus, atrophic gastritis in the antrum, and atrophic gastritis in both the corpus and antrum; the overall agreement with pathologic diagnosis was above 80% (351). However, this algorithm was not developed for pre-screening for pre-cancerous lesions or for screening for GC, but rather for delineating precancerous gastric lesions from one another.

Our results suggest that serum PGII and anti-*H. pylori* IgG combined (i.e., PGII ≥ 8.25 ng/ml plus anti-*H. pylori* IgG ≥ 24.02 EIU) may be useful for helping identify individuals who may require more invasive (i.e., gastroscopy) or intensive risk assessment or monitoring. PGII plus anti-*H. pylori* IgG is a logical combination because PGII is more sensitive to *H. pylori*-induced gastric inflammation than is PGI or the PGI/II ratio (503). Second, PGII may be more sensitive for detecting advanced gastric precancerous lesions in cases who have a reduction in or eradication of *H. pylori* infection (9). Finally, PGII may better capture the diffuse type of gastric adenocarcinomas that arise from non-atrophic gastric lesions (302-304). More studies are needed to confirm our results, and cost-effectiveness analyses would be needed before implementing combined serum PGII and anti-*H. pylori* IgG testing for prescreening high gastric cancer risk individuals for more invasive (i.e., gastroscopy) or intensive risk assessment or monitoring.

Our study had several limitations. First, a histopathological diagnosis was available on only 57% of the screening program participants, raising the possibility of selection bias; however, the distributions of sex, age, smoking, drinking, family history of

GC, *H. pylori* seroprevalence, and serum biomarker levels were similar between those who had a histopathological diagnosis and the full screening program participants (**Supplementary Table 3.1**). Second, because of practical considerations, gastrin-17 measurements were from fasting specimens although gastrin-17 levels after protein stimulation may be more informative. Finally, our study population was limited to persons in a particularly high-risk region in northern China, so caution should be taken in generalizing our results to other populations.

The strengths of our study are: (i) to our knowledge, it is the first study to report the accuracy of multiple serologic biomarkers (including serum PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG, and gastrin-17) in combination for identifying high gastric cancer risk individuals and screening for GC. (ii) The endoscopies and histopathological diagnoses were made blinded to the results of the serological tests, and vice versa. (iii) Histopathological diagnoses and serology were performed by the same study group according to consistent and standard protocols over the whole study period, which helps reduce misclassification bias and measurement errors. (iv) A natural population with a full spectrum of gastric diseases was used in our study, so our results are more likely to reflect the performance of these serum biomarkers in population-based screening practice.

In conclusion, our results suggest that serum PGII and anti-*H. pylori* IgG combined may be a useful aid for clinicians or large screening programs in identifying persons with abnormal gastric conditions that may require more invasive or intensive risk assessment or monitoring. Prescreening high gastric cancer risk individuals using non-invasive tests is of great importance, because prescreening could be used to substantially reduce unnecessary gastroscopies and associated harms and to help motivate and increase the compliance of patients for whom gastroscopies are warranted.

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Figure legends

Figure 3.1. Flow diagram of included and excluded participants

Figure 3.2. Receiver-operator characteristic curves of serum pepsinogen I (PGI), PGII, PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG individually and combined for discriminating between gastric conditions in the Zhuanghe Gastric Diseases Screening Program. (A) Abnormal conditions vs. normal mucosa (i.e., moderate/severe non-atrophic gastritis, atrophic gastritis/intestinal metaplasia, dysplasia, and gastric cancer vs. normal mucosa/mild non-atrophic gastritis). (B) Gastric cancer vs. gastric cancer-free (i.e., gastric cancer vs. normal mucosa/mild non-atrophic gastritis, moderate/severe non-atrophic gastritis, atrophic gastritis/intestinal metaplasia, and dysplasia).

Figure 3.3. Receiver-operator characteristic curves of serum pepsinogen I (PGI), PGII, PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG individually and combined for screening for gastric cancer (i.e., gastric cancer *vs.* normal mucosa/mild non-atrophic gastritis, moderate/severe non-atrophic gastritis, atrophic gastritis/intestinal metaplasia, and dysplasia) according to histological type in the Zhuanghe Gastric Diseases Screening Program. (A) The intestinal type of gastric adenocarcinoma, and (B) the diffuse type of gastric adenocarcinoma.

Table 3.1. Selected sociodemographic characteristics and gastric histopathological diagnoses of participants in the Zhuanghe Gastric Diseases Screening Program, China

Characteristics [a]	**Abnormal gastric conditions** [b] (n = 8,348)	**Normal/mild non-atrophic gastritis** (n = 2,287)
Male (%)	49.3	37.3
Age (yrs.)	50.9 ± 10.4	50.1 ± 10.1
Serum biomarkers		
PGI (ng/mL) [c]	101.7 ± 51.1	90.3 ± 45.3
PGII (ng/mL) [d]	15.0 ± 12.5	8.8 ± 8.7
PGI/II ratio [e]	9.8 ± 9.1	13.5 ± 9.1
Anti-*H. pylori* IgG, (EIU) [f]	43.2 ± 35.0	17.1 ± 21.0
Gastrin-17 (pmol/L) [g]	5.0 ± 10.5	3.2 ± 8.6
Histopathologies among participants with abnormal gastric conditions (%)		
Moderate/severe non-atrophic gastritis	53.3	
Atrophic gastritis/intestinal metaplasia	39.5	
Dysplasia	5.3	
Gastric cancer	1.3	
Unclassified	0.6	
GC histologic type among 109 gastric cancer patients (%)		
Intestinal adenocarcinoma	52.3	
Diffuse adenocarcinoma	43.1	
Other	4.6	
GC anatomic site among 103 gastric cancer patients (%)		
Antrum	40.8	
Angulus	11.7	
Body	28.2	
Cardia	6.8	
Multiple sites	12.6	

[a] Table reports % for categorical variables and mean ± standard deviation for continuous variables

[b] Moderate/severe non-atrophic gastritis, atrophic gastritis/intestinal metaplasia, dysplasia, gastric cancer

[c] Missing for 1 normal and 6 abnormal participants

[d] Missing for 6 normal and 15 abnormal participants

[e] Missing for 7 normal and 18 abnormal participants

[f] Missing for 81 normal and 228 abnormal participants

[g] Missing for 25 normal and 121 abnormal participants

Abbreviation: GC, gastric cancer

Table 3.2. Serum pepsinogen I (PGI), PGII, PGI/II ratio, anti-*H. pylori* IgG, and gastrin-17 levels in persons with different gastric histopathologies in the Zhuanghe Gastric Diseases Screening Program, China.

Biomarkers	**Normal mucosa/mild NAG** (n = 2,287)	**Moderate/severe NAG** (n = 4,450)	**AG/intestinal metaplasia** (n = 3,294)	**Dysplasia** (n = 444)	**GC** (n = 110)
PGI (ng/mL)	90.3 ± 0.9	103.3 ± 0.8	99.4 ± 0.9	105.3 ± 2.7	93.5 ± 5.1
PGII (ng/mL)	8.8 ± 0.2	14.6 ± 0.2	15.6 ± 0.2	15.7 ± 0.6	14.9 ± 1.0
PGI/II ratio	13.5 ± 0.2	10.4 ± 0.1	9.0 ± 0.1	9.5 ± 0.7	8.1 ± 0.6
Anti-*H. pylori* IgG (EIU)	17.1 ± 0.4	41.2 ± 0.5	46.5 ± 0.6	41.7 ± 1.8	38.5 ± 3.0
Gastrin-17 (pmol/L)	3.2 ± 0.2	5.0 ± 0.2	4.9 ± 0.2	5.8 ± 0.7	4.8 ± 1.3

Abbreviations: NAG, non-atrophic gastritis; AG, atrophic gastritis; GC, gastric cancer.

Table 3.3. Crude associations of serum biomarkers with abnormal gastric conditions[a] and gastric cancer, the Zhuanghe Gastric Diseases Screening Program, China

Serum biomarkers	Abnormal[a] vs. normal[b] gastric conditions				GC vs. GC-free			
	Abnormal (n)	Normal (n)	OR	95% CI	GC (n)	GC-free (n)	OR	95% CI
PGI (ng/mL)								
Quartile 1 (0 - 63.1)	1,719	574	1.00	N/A	32	2,262	1.00	N/A
Quartile 2 (63.2 - 86.0)	1,868	704	0.89	0.78, 1.01	20	2,554	0.55	0.32, 0.97
Quartile 3 (86.1 - 116.0)	2,169	545	1.33	1.16, 1.52	25	2,690	0.85	0.39, 1.11
Quartile 4 (116.1 - 645.4)	2,580	463	1.86	1.62, 2.13	33	3,012	0.77	0.48, 1.26
PGII (ng/mL)								
Quartile 1 (0 - 5.8)	1,516	863	1.00	N/A	18	2,361	1.00	N/A
Quartile 2 (5.9 - 9.5)	1,804	813	1.26	1.12, 1.42	28	2,589	1.42	0.78, 2.57
Quartile 3 (9.6 - 16.4)	2,262	411	3.13	2.74, 3.58	23	2,654	1.14	0.61, 2.11
Quartile 4 (16.5 - 238.4)	2,745	194	8.06	6.81, 9.53	41	2,900	1.85	1.06, 3.24
PGI/II ratio								
Quartile 1 (0 - 5.7)	2,483	191	7.29	6.17, 8.63	48	2,628	2.43	1.42, 4.14
Quartile 2 (5.8 - 8.9)	2,362	402	3.30	2.89, 3.77	24	2,744	1.16	0.64, 2.13
Quartile 3 (9.0 - 13.2)	1,848	772	1.34	1.20, 1.51	19	2,601	0.97	0.51, 1.84
Quartile 4 (13.3 - 421.8)	1,631	915	1.00	N/A	19	2,527	1.00	N/A
Anti-*H. pylori* IgG (EIU)								
Quartile 1 (-3.6 - 9.1)	1,558	1,111	1.00	N/A	17	2,665	1.00	N/A
Quartile 2 (9.2 - 26.1)	1,818	629	2.06	1.83, 2.32	33	2,429	2.13	1.19, 3.84
Quartile 3 (26.2 - 58.1)	2,127	334	4.54	3.95, 5.22	31	2,447	1.99	1.10, 3.61
Quartile 4 (58.2 - 196.1)	2,611	132	14.10	11.65, 17.07	29	2,725	1.67	0.91, 3.04
Gastrin-17 (pmol/L)								
Quartile 1 (0 - 0.1)	1,676	487	1.00	N/A	24	2,140	1.00	N/A
Quartile 2 (0.2 - 1.8)	2,076	853	0.71	0.62, 0.80	23	2,909	0.71	0.40, 1.25
Quartile 3 (1.9 - 4.7)	2,180	584	1.09	0.95, 1.24	40	2,724	1.31	0.79, 2.18
Quartile 4 (4.8 - 663.6)	2,289	338	1.97	1.69, 2.29	19	2,610	0.65	0.36, 1.19

[a] Includes moderate/severe non-atrophic gastritis, atrophic gastritis/intestinal metaplasia, dysplasia, gastric cancer
[b] Includes normal and mild non-atrophic gastric histologies
Abbreviations: GC, gastric cancer; OR, odds ratio; CI, confidence interval; PG, pepsinogen

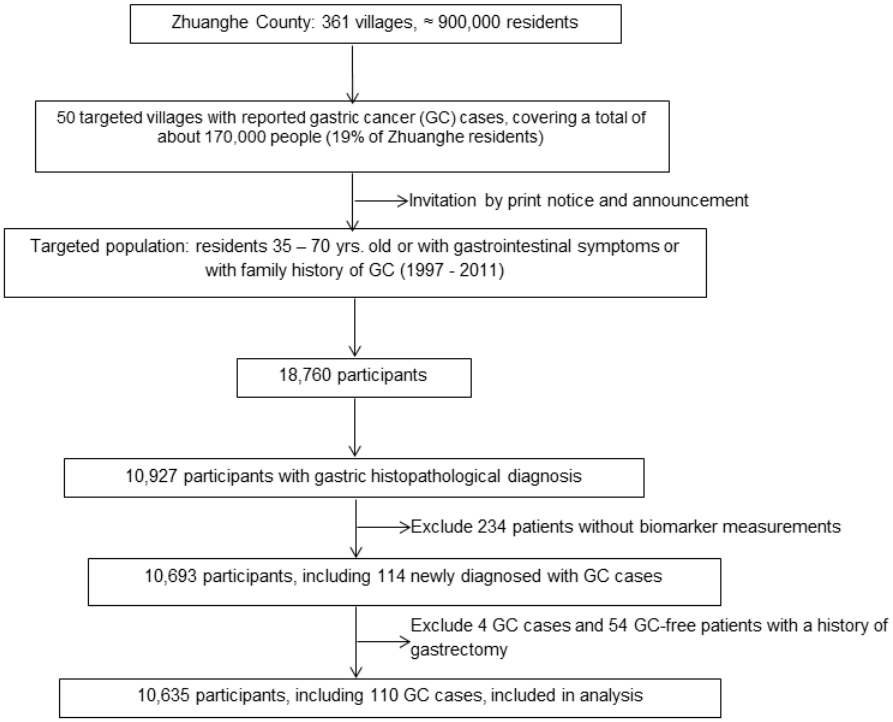

Figure 3.1. Flow diagram of included and excluded participants

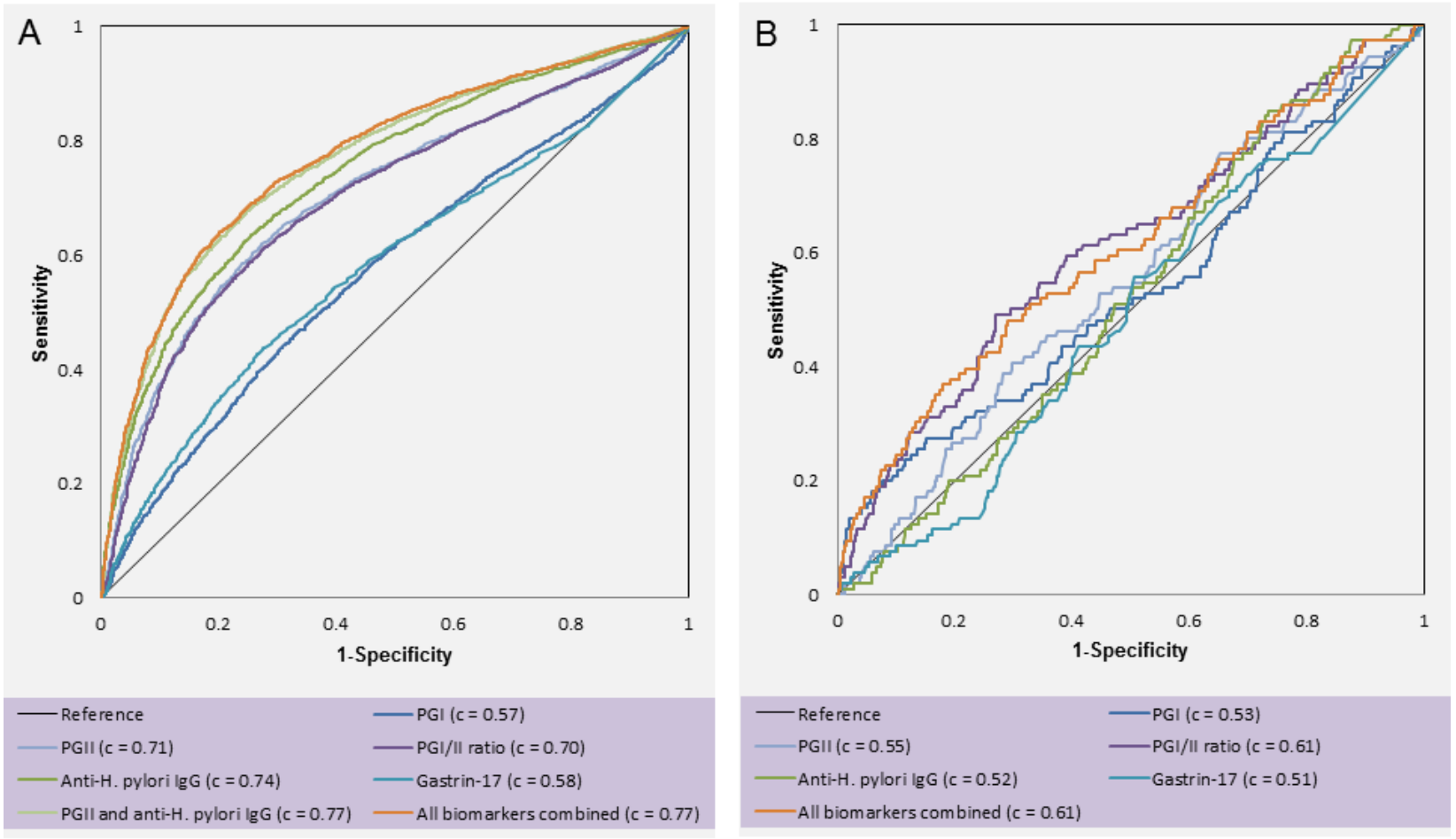

Figure 3.2. Receiver-operator characteristic curves of serum pepsinogen I (PGI), PGII, PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG individually and combined for discriminating between gastric conditions in the Zhuanghe Gastric Diseases Screening Program. (A) Abnormal conditions vs. normal mucosa (i.e., moderate/severe non-atrophic gastritis, atrophic gastritis/intestinal metaplasia, dysplasia, and GC vs. normal mucosa/mild non-atrophic gastritis). (B) Gastric cancer vs. gastric cancer-free (i.e., gastric cancer vs. normal mucosa/mild non-atrophic gastritis, moderate/severe non-atrophic gastritis, atrophic gastritis/intestinal metaplasia, and dysplasia).

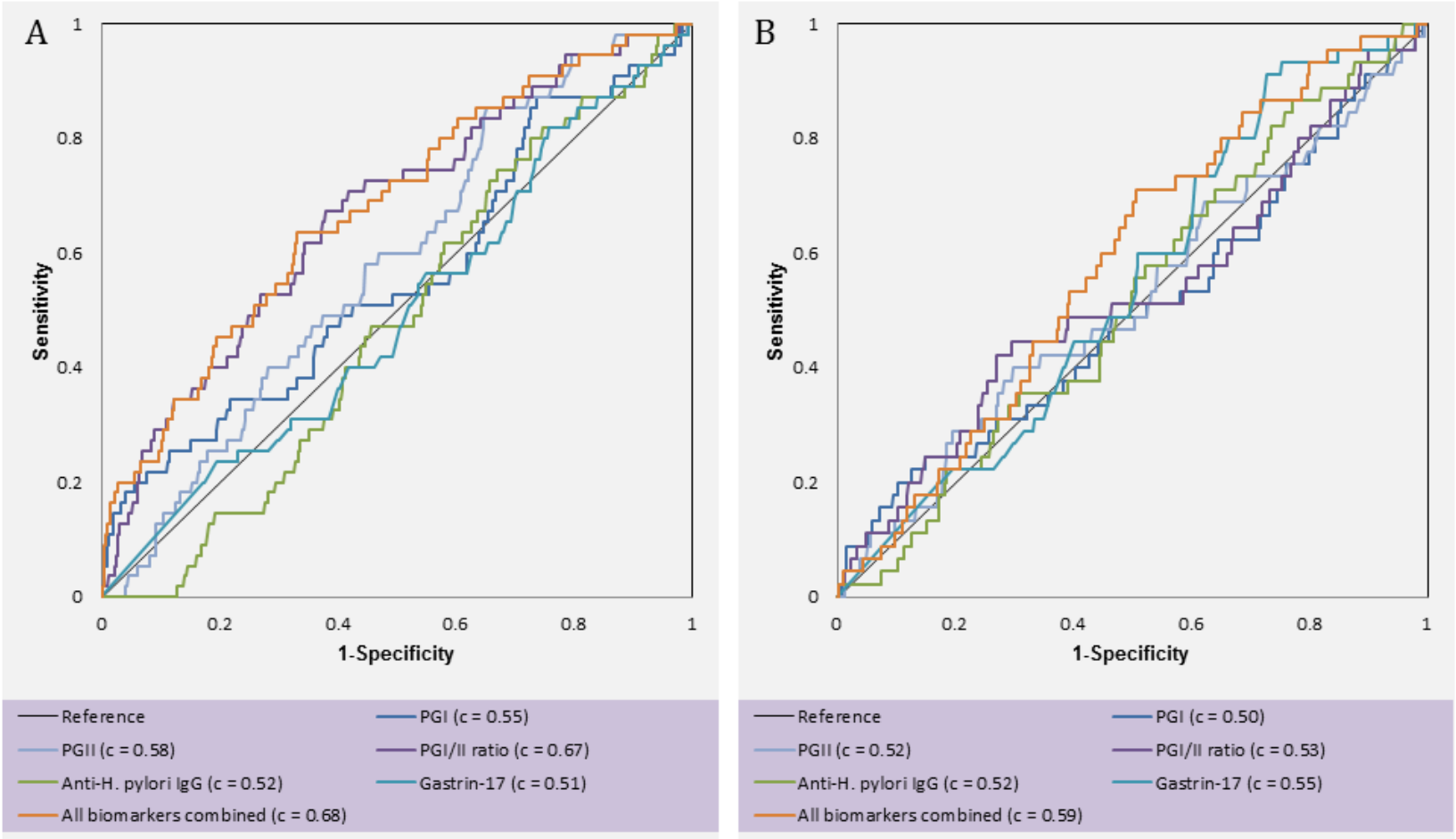

Figure 3.3. Receiver-operator characteristic curves of serum pepsinogen I (PGI), PGII, PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG individually and combined for screening for gastric cancer (i.e., gastric cancer *vs.* normal mucosa/mild non-atrophic gastritis, moderate/severe non-atrophic gastritis, atrophic gastritis/intestinal metaplasia, and dysplasia) according to histological type in the Zhuanghe Gastric Diseases Screening Program. (A) The intestinal type of gastric adenocarcinoma, and (B) the diffuse type of gastric adenocarcinoma.

Supplementary Table 3.1. Selected sociodemographic characteristics, risk behaviors, serum biomarker levels and pathological diagnosis of participants of the Zhuanghe Gastric Cancer Study, China.

Characteristics[a]	All participants (n=18,760)	Those included in analysis (n=10,635)
Male (%)	42.7	46.7
Age (yrs.)	49.9 ± 10.8	50.7 ± 10.4
Year at enrollment (%)		
1997 – 1999	21.5	24.3
2002 – 2004	40.4	32.8
2009 – 2011	38.1	43.0
Current smoker (%)		
Yes	22.9	23.1
No	66.1	59.7
Missing	11.0	17.2
Drink alcohol (%)		
Yes	15.3	16.1
No	73.8	66.7
Missing	11.0	17.2
Family history of GC (%)		
Yes	12.1	13.1
No	77.2	69.8
Missing	10.7	17.1
***H. pylori* seropositivity (%)**		
Positive	42.4	43.2
Negative	57.6	56.8
Serum biomarkers		
PGI	95.3 ± 47.4	99.2 ± 50.1
PGII	12.7 ± 11.1	13.7 ± 12.1
PGI/II ratio	10.7 ± 8.5	10.6 ± 9.2
H. *pylori* antibody IgG	36.9 ± 33.4	37.6 ± 34.2
Gastrin-17	4.7 ± 11.3	4.6 ± 10.1

[a] Table reports % for categorical variables and the mean ± standard deviation for continuous variables.

Chapter 4. Temporal Changes in Serum Biomarkers and Risk for Progression of Gastric Precancerous Lesions: a Longitudinal Study

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Running head: Serum markers for managing gastric precancerous lesions

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Abstract

Objective: Effectively managing precancerous lesions is crucial to reducing the gastric cancer (GC) burden. We evaluated associations of temporal changes in multiple serological markers (pepsinogen I [PGI], PGII, PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG) with risk for progression of gastric precancerous lesions.

Design: From 1997 to 2011, repeated esophagogastroduodenoscopies with gastric mucosal biopsies and blood sample collections were conducted on 2,039 participants (5,070 person-visits) in the Zhuanghe Gastric Diseases Screening Program, Liaoning, China. Serum biomarkers were measured using ELISA, and gastric biopsies were evaluated using standardized histologic criteria. Odds ratios (OR) and 95% confidence intervals (CI) were estimated using generalized estimating equations (GEE) for correlated binary outcomes.

Results: The ORs for progression of gastric conditions comparing those whose serum PGI, PGII, and anti-*H. pylori* IgG levels increased ≥ 50% relative to those whose decreased ≥ 50% were, respectively: 1.67 (CI, 1.22-2.28), 1.80 (CI, 1.40-2.33), and 1.93 (CI, 1.48-2.52). The OR for those whose PGII and anti-*H. pylori* IgG levels both increased ≥ 50% relative to those whose levels both decreased ≥ 50% was 3.18 (CI, 2.05-4.93), and for those whose PGI/II ratio decreased ≥ 50% relative to those whose increased ≥ 50% it was 1.40 (CI 1.08-1.81). Changes in gastrin-17 were not statistically significantly associated with progression.

Conclusion: These findings suggest that temporal changes in serum PGI, PGII, PGI/II ratio, and anti-*H. pylori* IgG levels (especially PGII and anti-*H. pylori* IgG combined) may be useful for assessing and managing risk for progression of gastric precancerous lesions.

KEYWORDS: Serological tests; management; gastric cancer; precancerous lesions

Introduction

Gastric cancer (GC) is the fourth most common incident cancer and second leading cause of cancer deaths worldwide, with 989,600 incident cases and 738,000 deaths in 2008 (1). GC, especially the intestinal type, is the end result of progression of precancerous lesions including non-atrophic gastritis, atrophic gastritis, intestinal metaplasia, and dysplasia (2-4, 66). This multi-step nature of gastric carcinogenesis provides unique opportunities for gastric cancer prevention and early detection, which is crucial to reducing the gastric cancer burden. It follows that effective management of precancerous lesions could lead to reduced gastric cancer incidence and mortality and could play an even more important role in reducing the gastric cancer burden than screening for gastric cancer itself, the value of which has already been well recognized.

Currently, there is no consensus on how to manage patients with gastric precancerous lesions. It has been stated that active surveillance is required for patients with precancerous lesions (9); however, gastric cancer develops in “only” 0.8% and 1.8% of patients with baseline atrophic gastritis or intestinal metaplasia within 10 years of follow-up, respectively (11); therefore, most persons with these lesions may not need multiple, expensive, invasive screening gastric endoscopies—which are not risk free—to prevent the disease. Of course, the problem is that we currently do not know which individuals fall into these categories, and markers are needed to stratify these patients according to their risk.

Serological markers are less invasive, more accessible, less expensive, and less time-consuming than are markers in tissues, such as those obtained at endoscopy. Currently available serological markers include pepsinogens I and II (PGI and PGII), gastrin-17, and anti-*Helicobacter pylori* (*H. pylori*) antibody (499, 511). Cross-sectional

studies suggested that levels of these markers were correlated with gastric conditions (295-298, 323, 325, 351, 503, 510, 512, 513); therefore, monitoring temporal changes in the markers may help identify high gastric cancer risk individuals whose precancerous lesions are more likely to progress. However, to date, no longitudinal study has evaluated whether temporal changes in PGs, gastrin-17, and anti-*H. pylori* antibody levels are associated with progression of gastric precancerous lesions.

To assess the potential for monitoring changes in serum PGs, gastrin-17, and anti-*H. pylori* antibody levels for assessing and managing risk for gastric precancerous lesion progression, we analyzed longitudinal data from a large gastric diseases screening program in a high risk population in China.

Materials and Methods

Study population

This study was approved by the Human Ethics Review Committee of the First Affiliated Hospital of China Medical University (Shenyang, China). Written informed consent was obtained from each participant in accordance with the Declaration of Helsinki and its later revision.

Our study population was from the Zhuanghe Gastric Diseases Screening Program, a population-based, combined serologic/endoscopic screening program for gastric diseases, particularly GC, that has been conducted in Zhuanghe County, a high gastric cancer risk area in China (514), since 1997. The study population selection and recruitment process was reported previously (512). Briefly, the screening program targets all residents who are 35 to 70 years old or who have gastrointestinal symptoms (including abdominal bloating, heartburn, acid reflux, nausea, hiccups, belching, decreased appetite, and stomachache) or a positive family history of gastric cancer in 50

selected villages, which represent Zhuanghe County geographically. Participation is voluntary, and to date, 18,760 participants have been recruited, and baseline endoscopic examinations with mucosal biopsies and blood sample collection were conducted on 10,635 participants. For those enrolled from 1997 to 1999, follow-up endoscopic examinations were recommended for all participants; for those enrolled after 1999, follow-up endoscopic examinations were only recommended for those with precancerous lesions. So far, 2,336 participants have had at least one follow-up endoscopic examination with mucosal biopsies and blood sample collection, resulting in a total of 6,043 person-visits. After excluding those without histopathological diagnoses (n = 194) or biomarker measurements (n = 89) and those who were diagnosed with gastric cancer at baseline (n = 14), 2,039 participants (5,070 person-visits) were included in the final analysis.

Serological measurements

A 5 ml fasting venous blood sample was collected at each person's visit. All samples were centrifuged immediately at 3,500×g for 10 minutes, and a serum aliquot was immediately frozen and stored until analysis. Serum PGI, PGII, gastrin-17, and anti-*H. pylori* IgG were measured using enzyme-linked immunosorbent assays (Pepsinogen I ELISA; Pepsinogen II ELISA; Gastrin-17 ELISA kit; and *Helicobacter pylori* IgG ELISA; BIOHIT Plc, Helsinki, Finland) according to the manufacturer's protocols, blinded to the histopathological diagnosis. Samples that yielded implausible values were re-tested. Duplicate negative and positive controls were included in each 96-well plate. The mean intra-assay coefficients of variation (CV) were 11% for PGI, 12% for PGII, 15% for gastrin-17, and 11% for anti-*H. pylori* IgG.

Endoscopic and histopathological examinations

Experienced endoscopists blinded to the patients' serological test results performed the gastrointestinal endoscopies. Mucosal biopsies were obtained from the gastric body, angulus, antrum, and, if applicable, lesion site. The biopsies were oriented, fixed in 95% ethanol, embedded in paraffin blocks, and then sectioned and stained with hematoxylin and eosin in local study centers. Each stained section was independently evaluated by two gastrointestinal pathologists using standard criteria from the WHO classification for gastric cancer (500) and the visual analog scale of the updated Sydney System for gastritis (501). For histologic sections on which there was initial disagreement on the histopathologic interpretation, the final results were determined through adjudication among the two pathologists and a third pathologist. Each participant was assigned a global diagnosis based on the most severe lesion found among all the biopsy specimens. Accordingly, the 5,070 person-visits with a histopathologic diagnosis were classified as: normal mucosa/mild non-atrophic gastritis (n = 850), moderate non-atrophic gastritis (n = 1647), severe non-atrophic gastritis (n = 1504), mild atrophic gastritis (n = 147), moderate atrophic gastritis (n = 502), severe atrophic gastritis (n = 233), low grade dysplasia (n = 171), high grade dysplasia (n = 6), and gastric cancer (n = 10).

Statistical analysis

All statistical analyses were performed using SAS 9.3 statistical software (SAS Institute Inc., Cary, NC, USA). A *P* value ≤ 0.05 (two-sided) was considered statistically significant.

Temporal changes in serum biomarker levels at follow-up visits were calculated as proportional changes relative to the baseline levels (i.e., [follow-up - baseline]/baseline x 100%) to account for interpersonal variations in the baseline and

changes in serum biomarker levels. To determine the progression status at each follow-up visit, each participant was assigned a global severity score at baseline (A) and follow-up/s (B) according to a commonly used nine-category score system which defines the gastric premalignant process (515-519): 1 for normal mucosa/mild non-atrophic gastritis, 2 for moderate non-atrophic gastritis, 3 for severe non-atrophic gastritis, 4 for mild atrophic gastritis, 5 for moderate atrophic gastritis, 6 for severe atrophic gastritis, 7 for low grade dysplasia, 8 for high grade dysplasia, and 9 for GC. We subtracted score A from score B to determine the progression status at each follow-up visit. If the difference between score B and A was > 0, the progression status at this follow-up visit was defined as progression, otherwise it was defined as no progression.

The odds ratios (OR) with 95 percent confidence intervals (95% CI) were calculated as measures of association. Since some participants (n = 778) had more than one follow-up visit, generalized estimating equations (GEE) were used to account for the correlated nature of the binary outcome. Since the goal of the present study was not about etiology but rather to assess the potential prediction ability of temporal changes in serum biomarkers for risk of progression of gastric precancerous lesions, in our primary analysis we did not include covariates in the model; however, as a sensitivity analysis, we included age and sex in the model to assess the potential prediction ability of temporal changes in serum biomarkers beyond these two basic variables. Also, we conducted stratified analyses by selected baseline characteristics (sex, age, baseline histopathologic conditions, and baseline serological test results) to assess the potential prediction ability of the serum biomarkers in population subgroups.

Results

Selected characteristics of the study participants according to sex are summarized in **Table 4.1**. About half (53.2%) of the participants were males, and the mean age was 49.8 (± STD 10.5) years. The majority of the participants was enrolled between 1997 and 1999, had moderate/severe non-atrophic gastritis at enrollment, and had one follow-up visit. The median follow-up time was 2.3 (range: 0.4 - 7.6) years among males and 2.2 (range: 0.4 - 7.6) years among females.

Associations of temporal changes in serum biomarkers with histologic progression

Associations of temporal changes in serum PGI, PGII, the PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG levels with progression of gastric precancerous lesions are shown in **Table 4.2**. Those whose PGI or PGII levels increased ≥ 50%, relative to those whose PGI or PGII levels decreased ≥ 50%, had statistically significant 67% or 80% higher odds of progression of gastric conditions, respectively. Those whose PGI/II ratio decreased ≥ 50% relative to those whose PGI/II ratio increased ≥ 50% had statistically significant 40% higher odds of progression. Those whose gastrin-17 levels increased ≥ 500% relative to those whose gastrin-17 levels decreased ≥ 100% had 33% ($p = 0.08$) higher odds of progression. Relative to those whose anti-*H. pylori* IgG titers decreased ≥ 50%, those whose anti-*H. pylori* IgG titers decreased 20-50%, remained within 20%, increased 20-50%, or increased ≥ 50% had 21%, 58%, 64%, and 93% higher odds of progression (*P* for trend < 0. 01), respectively. After controlling for age and sex, the results were essentially unchanged (**Supplementary Table 4.1**).

We conducted multiple sensitivity analyses. First, the global severity score at each visit was assigned according to a four-category score system, which does not consider the severity of non-atrophic gastritis or atrophic gastritis: 1 for normal

mucosa/mild non-atrophic gastritis, 2 for mild and severe non-atrophic gastritis, 3 for atrophic gastritis, and 4 for GC. The results (**Supplementary Table 4.2**) were similar to those above using the nine-category score system. Second, instead of using serum biomarker level relative changes, we used their absolute changes (**Supplementary Table 4.3**) and rates of change (i.e., absolute changes/time, **Supplementary Table 4.3**) as predictors for progression, and the results were similar to those reported above. Third, instead of comparing all follow-up visits with the baseline visit, we compared each follow-up visit with the previous visit (**Supplementary Table 4.5**), the second visit with the baseline visit (**Supplementary Table 4.6**), and the last visit with the baseline visit (**Supplementary Table 4.7**), and the results were also similar to those reported above.

Associations of temporal changes in serum biomarkers with histologic progression according to selected baseline characteristics

We also investigated associations of temporal changes in the serum biomarkers with histologic progression according to baseline histopathologic conditions (normal mucosa/mild non-atrophic gastritis *vs*. mild/severe superficial gastritis). We do not present results limited to patients with more advanced baseline lesions (i.e., atrophic gastritis and dysplasia) because of the insufficient power. As shown in **Table 4.3**, the directions of the associations between the strata were similar but somewhat stronger among those with normal mucosa/mild non-atrophic gastritis at baseline. When we stratified the results by sex or age (< 55 *vs*. ≥ 55 years old), we found the associations to be slightly stronger among females and younger participants (data not shown), possibly because females and younger participants tended to have less severe baseline gastric lesions.

In addition, because our previous cross-sectional study (manuscript under review) suggested that serum levels of PGII ≥ 8.3 ng/ml plus anti-*H. pylori* IgG ≥ 24.0 EIU may be useful for identifying high gastric cancer risk individuals, we investigated associations of temporal changes in the serum biomarkers with progression of gastric precancerous lesions stratified by baseline serological test results (i.e., PGII ≥ 8.3 ng/ml or anti-*H. pylori* IgG ≥ 24.0 EIU *vs.* otherwise). As shown in **Table 4.4**, the associations among those with an abnormal baseline serological test (i.e., PGII ≥ 8.3 ng/ml or anti-*H. pylori* IgG ≥ 24.0 EIU) were similar to, but slightly weaker than, the non-stratified associations; the associations among those with an abnormal baseline serological test were similar to those among those with a normal baseline serological test except that the association of anti-*H. pylori* IgG titers with histologic progression was somewhat stronger than among those with normal baseline serological tests.

Associations of temporal changes in serum PGII and anti-*H. pylori* IgG combined with histologic progression

Since temporal changes in serum PGII and anti-*H. pylori* IgG levels individually were the most strongly associated with progression of gastric precancerous lesions, we investigated the association of temporal changes of the two in combination with progression. As shown in **Table 4.5**, relative to those whose PGII levels and anti-*H. pylori* IgG titer both decreased ≥ 50%, those whose PGII levels remained within 50% and anti-*H. pylori* IgG titer increased ≥ 50%, those whose PGII levels increased 50% and anti-*H. pylori* IgG titer remained within 50%, and those whose PGII levels and anti-*H. pylori* IgG titer both increased ≥ 50% had statistically significant 108%, 87%, and 218% higher odds of progression, respectively.

Serum PGI, PGI/II ratio, and gastrin-17 levels across different site-specific gastric conditions

Because PGI and gastrin-17 production is site-specific (i.e., PGI in the body and gastrin-17 in the antrum), we investigated associations of serum PGI, the PGI/II ratio, and gastrin-17 levels with site-specific gastric conditions. Serum PGI, PGI/II ratio, and gastrin-17 levels in persons with different gastric histopathologies in the body or in the antrum are shown in **Figure 4.1**. Across the histopathologic conditions in the body from normal mucosa/mild non-atrophic gastritis, moderate/severe non-atrophic gastritis, to atrophic gastritis, PGI (Panel A) first increased, peaked at moderate/severe non-atrophic gastritis, and then decreased substantially; the PGI/II ratio (Panel B) monotonically decreased; and gastrin-17 monotonically increased (Panel C). Across the histopathologic conditions in the antrum from normal mucosa/mild non-atrophic gastritis, moderate/severe non-atrophic gastritis, to atrophic gastritis, PGI (Panel D) first increased, peaked at moderate/severe non-atrophic gastritis, and then decreased slightly; the PGI/II ratio (Panel E) first decreased, bottomed at moderate/severe non-atrophic gastritis, and then slightly increased; and gastrin-17 (Panel F) monotonically decreased.

Discussion

In this large longitudinal study, we found that an increase in serum PGI, PGII, anti-*H. pylori* IgG levels (especially PGII and anti-*H. pylori* IgG combined) and a decrease in the PGI/II ratio were associated with risk for progression of gastric precancerous lesions, especially among those with normal mucosa/mild non-atrophic gastritis at baseline, suggesting that monitoring serum PGs and anti-*H. pylori* IgG levels has potential for assessing and managing risk for gastric precancerous conditions. To our knowledge, this is the first reported study to have investigated temporal changes in these markers, individually or collectively, in relation to gastric cancer prevention.

Effective management of gastric premalignant conditions is crucial to reducing gastric cancer incidence and mortality (520). Currently, there is no consensus on how to manage patients with precancerous lesions. According to the most recent recommendations from European expert panels (9, 520), active surveillance is required for patients with precancerous lesions. However, gastric cancer risk is too “low” to justify endoscopic surveillance on all patients with precancerous lesions due to cost-effectiveness considerations (520), and markers to further stratify gastric cancer risk among those patients are needed. Risk stratification guided endoscopic surveillance of gastric premalignant conditions is of great importance, because it could substantially reduce unnecessary gastroscopies and associated harms by allocating limited resources to high gastric cancer risk individuals.

Serum PGI, PGII, the PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG are promising markers (9, 499), and multiple cross-sectional studies have investigated their relations to gastric conditions (295-298, 323, 325, 351, 503, 510, 512, 513). Also, multiple population-based studies in Japan evaluated the accuracy of serum PGs for screening for GC, yielding mixed results (505-508). In addition, many follow-up studies found that

serum PGI, PGII, the PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG levels measured once at baseline were associated with future gastric cancer risk [e.g., (300-305, 308, 311-314, 316, 318, 509)]. Based on currently available evidence, it has been proposed that these serological biomarkers might be useful for identifying those with precancerous gastric lesions who should be referred for gastroscopy (9, 499). All previous studies were focused on single baseline absolute levels of serum PGI, PGII, the PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG for screening for gastric cancer or for identifying high gastric cancer risk individuals for diagnostic gastroscopy, and none reported investigating the potential role of monitoring changes in these serological biomarkers over time for gastric cancer prevention.

As reported herein, we found that an increase in serum PGI or PGII and a decrease in the PGI/II ratio were associated with progression of the most severe identified gastric lesion in the whole stomach, especially for those with normal mucosa/mild non-atrophic gastritis at baseline, and among the three PG-related markers, the association of PGII with progression was the strongest. The distribution of PGII-producing cells includes the entire stomach and the duodenum (292-294), so its change was more likely to represent abnormal histologic progression in the whole stomach. Also, PGII is more sensitive to *H. pylori*-induced gastric inflammation than is PGI or the PGI/II ratio (503). Since PGI is only produced in the glandular mucosa in the body of the stomach, we also examined associations of serum PGI and the PGI/II ratio with site-specific histopathologic conditions (i.e., histopathologic conditions in the body and in the antrum). Our results suggested that a decrease in serum PGI or the PGI/II ratio only indicated atrophy in the body, while serum PGI only decreased slightly and the PGI/II ratio actually increased in the presence of atrophy in the antrum. Taken together, an increase in serum PGI and PGII levels and a decrease in the PGI/II ratio indicated

progression of the most severe gastric lesion in the whole stomach, especially among patients with normal mucosa/mild non-atrophic gastritis at baseline; however, a decrease in serum PGI could indicate regression from non-atrophic gastritis to normal mucosa or progression from non-atrophic gastritis to atrophic gastritis in the body, and information on other serological biomarkers is needed to determine which is more likely to be true.

We found that change in serum gastrin-17 levels was not substantially associated with progression of the most severe gastric lesion in the whole stomach. Gastrin-17 is released by G cells in the antrum. Serum gastrin-17 decreases when the number of G cells in the antrum decreases or when the intra-gastric acidity is high (320), which makes changes in serum gastrin-17 levels difficult to interpret; consistent with this belief, our results suggested that serum gastrin-17 levels decreased slightly in the presence of atrophy in the antrum and increased in the presence of atrophy in the body.

We found that an increase in serum anti-*H. pylori* IgG level was associated with progression of gastric precancerous lesions, especially among patients with baseline normal mucosa/mild non-atrophic gastritis. These longitudinal results are consistent with our previous cross-sectional findings that serum anti-*H. pylori* IgG antibody titer was positively correlated with grade of histological gastritis and mucosal bacterial density (512). Furthermore, a recent follow-up study showed that seropositivities for *H. pylori*-specific antibodies for CagA and GroEL were associated with progression of gastric precancerous lesions. In addition, intervention trials showed that *H. pylori* eradication reduced risk of progression (515, 517, 521-524).

Our previous cross-sectional study results (manuscript under review) suggested that serum PGII combined with anti-*H. pylori* IgG was useful for identifying individuals with abnormal gastric histology. In the present study, we found that individuals who had

serum levels of PGII ≥ 8.3 ng/ml or anti-*H. pylori* IgG ≥ 24.0 EIU at baseline and had temporal increases in both markers were at increased risk for progression of gastric lesions. Taken together, our previous and present results suggest that the combination of serum PGII and anti-*H. pylori* IgG levels could be useful for identifying and then monitoring individuals at risk for GC. However, additional biomarkers (e.g., tissue markers obtained at endoscopy) need to be identified and incorporated into the panel to further improve the ability to predict clinically significant histologic progression and the need for and timing of follow-up endoscopy.

Our study had several limitations. First, not all screening program participants were endoscopically followed and included in the analysis, raising the possibility of selection bias; however, the distributions of sex, age, smoking, drinking, family history of gastric cancer were similar between those who were included in this study and the full screening program participants (**Supplementary Table 4.8**). Second, because of practical and ethical considerations, we did not take biopsies from both the antrum and the body on all participants if the endoscopists determined that the gastric mucosa in a site was normal. This limited our power to investigate whether changes in biomarkers predicted site-specific progression of precancerous lesions; however, this would not have affected our main outcome, progression of the most severe gastric lesion in the whole stomach, which was based on the most severe lesion found among all the biopsy specimens. Third, our sample size for those with more advanced baseline lesions (i.e., atrophic gastritis and dysplasia) that progressed was insufficient to adequately assess associations among this subgroup of participants. Finally, our study population was limited to persons in a particularly high-risk region in northern China, so caution should be taken in generalizing our results to other populations.

The strengths of our study are: (i) To our knowledge, it is the first study to examine whether temporal changes in serum PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG, and gastrin-17 are associated with risk for progression of gastric precancerous lesions. (ii) The endoscopies and histopathological diagnoses were made blinded to the results of the serological tests, and vice versa. (iii) Histopathological diagnoses and serology were performed by the same study group according to consistent and standard protocols over the whole study period, which helps reduce misclassification bias and measurement errors. (iv) With the longitudinal design of our study, we were able to calculate relative changes of serum biomarkers over time, which helps control for baseline and temporal interpersonal serum biomarker level variation.

In conclusion, the results from this large longitudinal study suggest that an increase in serum PGI, PGII, anti-*H. pylori* IgG levels and an decrease in the PGI/II ratio may be associated with progression of gastric precancerous lesions, especially among those with normal mucosa/mild non-atrophic gastritis at baseline. Also, our present results, taken together with our previous results, suggest that the combination of serum PGII and anti-*H. pylori* IgG could be used to identify and monitor individuals at increased risk for GC.

Competing interests: None

Data sharing statement: We are happy to share our additional unpublished data on other blood biomarkers from this study with interested researchers through collaboration.

Figure Legend:

Figure 4.1. Serum pepsinogen I (PGI), PGI/II ratio, and gastrin-17 levels in persons with different gastric histopathologies in the Zhuanghe Gastric Diseases Screening Program, China. (A). Serum PGI levels in persons with different histopathologies in the gastric body; (B). Serum PGI/II ratio in persons with different histopathologies in the gastric body; (C). Serum gastrin-17 levels in persons with different gastric histopathologies in the gastric body; (D). Serum PGI levels in persons with different histopathologies in the gastric antrum; (E). Serum PGI/II ratio in persons with different histopathologies in the gastric antrum; (F). Serum gastrin-17 levels in persons with different histopathologies in the gastric antrum.

Abbreviations: NAG, non-atrophic gastritis; AG, atrophic gastritis.

Table 4.1. Selected characteristics of participants in the Zhuanghe Gastric Diseases Screening Program, China.

Characteristics [a]	**Males** (n = 1,085)	**Females** (n = 954)
Age (yrs.)	50.8 ± 11.0	48.8 ± 9.8
Year at enrollment (%)		
1997 - 1999	70.2	65.6
2002	8.9	10.5
2008 - 2010	20.8	23.9
Serum biomarker levels		
PGI (ng/mL)	109.3 ± 57.1	92.6 ± 47.7
PGII (ng/mL)	17.3 ± 14.0	14.0 ± 10.9
PGI/II ratio	9.1 ± 9.1	9.9 ± 10.0
Gastrin-17 (pmol/L)	3.5 ± 9.7	4.3 ± 13.1
Anti-*H. pylori* IgG (EIU)	42.0 ± 33.9	40.4 ± 32.6
Baseline histopathologies (%)		
Normal mucosa/mild non-atrophic gastritis	14.0	18.1
Moderate non-atrophic gastritis	22.8	27.6
Severe non-atrophic gastritis	33.7	34.5
Mild atrophic gastritis	4.5	4.4
Moderate atrophic gastritis	10.7	7.0
Severe atrophic gastritis	7.0	4.3
Low grade dysplasia	7.2	3.8
High grade dysplasia	0.1	0.3
Median (range) length of follow-up time (yrs.)	2.3 (0.4 - 7.6)	2.2 (0.4 – 7.6)
Number of follow-up visits (%)		
1	60.8	63.0
2	28.5	30.1
3	8.4	6.2
4	2.3	0.7

[a] Mean ± STD, unless otherwise indicated.
Abbreviations: *H. pylori*: *Helicobacter pylori*; PG: pepsinogen

Table 4.2. Associations of relative temporal changes in serum PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG, and gastrin-17 levels with progression of gastric precancerous lesions; Zhuanghe Gastric Diseases Screening Program, China.

Relative change [a]	Progression (n)	No progression (n)	OR [b]	95% CI		*P* for trend
Serum PGI						
Decreased ≥ 50%	97	402	1.00	N/A		
Decreased 20 - 50%	148	637	0.97	0.72	1.30	
Within 20%	195	678	1.21	0.91	1.60	< 0.01
Increased 20 - 50%	101	303	1.50	1.09	2.06	
Increased ≥ 50%	123	323	1.67	1.22	2.28	
Serum PGII						
Decreased ≥ 50%	114	589	1.00	N/A		
Decreased 20 - 50%	135	442	1.53	1.17	1.99	
Within 20%	147	503	1.52	1.16	2.00	< 0.01
Increased 20 - 50%	64	242	1.35	0.97	1.87	
Increased ≥ 50%	200	561	1.80	1.40	2.33	
Serum PGI/II ratio						
Increased ≥ 50%	171	698	1.00	N/A		
Increased 20 - 50%	76	254	1.20	0.89	1.62	
Within 20%	148	503	1.19	0.94	1.51	0.07
Decreased 20 - 50%	111	454	0.98	0.75	1.28	
Decreased ≥ 50%	152	409	1.40	1.08	1.81	
Serum gastrin-17						
Decreased ≥ 100%	86	319	1.00	N/A		
Decreased 20 to 100%	106	378	0.97	0.70	1.36	
Within 20%	150	697	0.83	0.61	1.14	0.02
Increased 20 - 500%	162	501	1.15	0.85	1.55	
Increased ≥ 500%	112	309	1.33	0.96	1.85	
Serum anti-*H. pylori* IgG						
Decreased ≥ 50%	114	577	1.00	N/A		
Decreased 20 - 50%	83	367	1.21	0.90	1.63	
Within 20%	140	453	1.58	1.19	2.08	< 0.01

Increased 20 - 50%	63	197	1.64	1.15	2.34
Increased ≥ 50%	206	528	1.93	1.48	2.52

[a] Defined as: (follow-up – baseline)/baseline x 100%
[b] Estimated using generalized estimating equations (GEE) for correlated binary outcomes
Abbreviations: *H. pylori*: *Helicobacter pylori*; PG: pepsinogen

Table 4.3. Associations of relative temporal changes in serum PGI, PGII, the PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG levels with progression of gastric precancerous lesions stratified by baseline histopathologic conditions; Zhuanghe Gastric Diseases Screening Program, China.

Relative change [a]	Normal mucosa/mild non-atrophic gastritis at baseline (n = 325)				Mild/severe superficial gastritis at baseline (n = 1,205)			
	OR [b]	95% CI		*P* for trend	OR [b]	95% CI		*P* for trend
Serum PGI								
Decreased ≥ 50%	1.00	N/A			1.00	N/A		
Decreased 20 - 50%	0.95	0.56	1.62		1.12	0.73	1.71	
Within 20%	1.30	0.79	2.12	< 0.01	1.43	0.96	2.14	< 0.01
Increased 20 - 50%	1.71	0.91	3.25		1.89	1.20	2.98	
Increased ≥ 50%	2.05	1.16	3.64		1.85	1.19	2.87	
Serum PGII								
Decreased ≥ 50%	1.00	N/A			1.00	N/A		
Decreased 20 - 50%	1.30	0.75	2.28		1.50	1.05	2.13	
Within 20%	2.00	1.10	3.66	< 0.01	1.55	1.09	2.21	0.44
Increased 20 - 50%	1.39	0.65	3.00		1.31	0.86	2.01	
Increased ≥ 50%	2.65	1.58	4.45		1.22	0.86	1.73	
Serum PGI/II ratio								
Increased ≥ 50%	1.00	N/A			1.00	N/A		
Increased 20 - 50%	1.80	0.91	3.59		0.98	0.65	1.47	
Within 20%	1.60	0.89	2.87	0.03	1.02	0.74	1.41	0.19
Decreased 20 - 50%	1.63	0.92	2.91		0.79	0.56	1.12	
Decreased ≥ 50%	1.85	1.12	3.05		0.84	0.58	1.22	
Serum gastrin-17								
Decreased ≥ 100%	1.00	N/A			1.00	N/A		
Decreased 20 to 100%	0.85	0.41	1.73		1.19	0.78	1.83	
Within 20%	1.32	0.70	2.47	< 0.01	0.70	0.46	1.07	0.81
Increased 20 - 500%	1.98	1.06	3.69		1.13	0.75	1.70	
Increased ≥ 500%	2.33	1.20	4.54		1.07	0.67	1.70	

Serum anti-*H. pylori* IgG								
Decreased ≥ 50%	1.00	N/A			1.00	N/A		
Decreased 20 - 50%	3.10	1.55	6.19		1.02	0.68	1.53	
Within 20%	4.60	2.30	9.22	< 0.01	1.62	1.13	2.32	0.05
Increased 20 - 50%	3.64	1.56	8.54		1.49	0.94	2.36	
Increased ≥ 50%	3.43	2.01	5.86		1.32	0.92	1.90	

[a] Defined as: (follow-up – baseline)/baseline x 100%
[b] Estimated using generalized estimating equations (GEE) for correlated binary outcomes
Abbreviations: *H. pylori*: *Helicobacter pylori*; PG: pepsinogen

Table 4.4. The associations of relative temporal changes in serum PGI, PGII, the PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG levels with progression of gastric precancerous lesions stratified by baseline serological test results; Zhuanghe Gastric Diseases Screening Program, China.

Relative change [a]	Abnormal baseline biomarker tests [b] (n = 1,612)				Normal baseline biomarker tests [c] (n = 363)			
	OR [d]	95% CI		*P* value	OR [a]	95% CI		*P* value
Serum PGI								
Decreased ≥ 50%	1.00	N/A			1.00	N/A		
Decreased 20 - 50%	1.02	0.73	1.43		0.73	0.36	1.47	
Within 20%	1.34	0.97	1.86	< 0.01	0.85	0.46	1.55	0.05
Increased 20 - 50%	1.55	1.07	2.24		1.06	0.51	2.19	
Increased ≥ 50%	1.58	1.09	2.30		1.61	0.86	3.03	
Serum PGII								
Decreased ≥ 50%	1.00	N/A			1.00	N/A		
Decreased 20 - 50%	1.50	1.13	1.99		1.48	0.53	4.12	
Within 20%	1.45	1.09	1.95	< 0.01	1.62	0.59	4.44	0.10
Increased 20 - 50%	1.42	1.00	2.03		1.17	0.40	3.43	
Increased ≥ 50%	1.61	1.20	2.16		1.93	0.79	4.73	
Serum PGI/II ratio								
Increased ≥ 50%	1.00	N/A			1.00	N/A		
Increased 20 - 50%	1.07	0.77	1.48		2.81	1.21	6.51	
Within 20%	1.19	0.92	1.54	0.21	1.29	0.60	2.79	0.87
Decreased 20 - 50%	0.86	0.63	1.17		1.56	0.77	3.19	
Decreased ≥ 50%	1.42	1.04	1.93		1.38	0.69	2.72	
Serum gastrin-17								
Decreased ≥ 100%	1.00	N/A			1.00	N/A		
Decreased 20 to 100%	1.02	0.71	1.46		0.81	0.34	1.93	
Within 20%	0.77	0.54	1.09	0.16	0.92	0.46	1.85	0.13
Increased 20 - 500%	1.18	0.84	1.65		1.04	0.53	2.06	
Increased ≥ 500%	1.19	0.81	1.75		1.51	0.73	3.09	

Serum anti-*H. pylori* IgG								
Decreased ≥ 50%	1.00	N/A			1.00	N/A		
Decreased 20 - 50%	1.10	0.80	1.51		2.48	1.04	5.91	
Within 20%	1.46	1.09	1.97	< 0.01	3.42	1.49	7.84	0.02
Increased 20 - 50%	1.49	1.01	2.19		3.33	1.25	8.83	
Increased ≥ 50%	1.87	1.38	2.52		2.60	1.25	5.40	

[a] Defined as: (follow-up – baseline)/baseline x 100%
[b] PGII ≥ 8.3 ng/ml or anti-*H. pylori* IgG ≥ 24.0 EIU
[c] PGII < 8.3 ng/ml and anti-*H. pylori* IgG < 24.0 EIU
[d] Estimated using generalized estimating equations (GEE) for correlated binary outcomes
Abbreviations: *H. pylori*: *Helicobacter pylori*; PG: pepsinogen

Table 4.5. Associations of temporal changes in serum PGII and anti-*H. pylori* IgG in combination with progression of gastric precancerous lesions; Zhuanghe Gastric Diseases Screening Program, China.

Relative change [a]	**Progression (n)**	**No progression (n)**	**OR** [b]	**95% CI**	
PGII decreased ≥ 50% and anti-*H. pylori* IgG decreased ≥ 50%	40	223	1.00	N/A	
PGII decreased ≥ 50% and anti-*H. pylori* IgG within 50%	47	204	1.40	0.88	2.23
PGII decreased ≥ 50% and anti-*H. pylori* IgG increased ≥ 50%	17	95	1.10	0.60	2.01
PGII within 50% and anti-*H. pylori* IgG decreased ≥ 50%	54	240	1.33	0.85	2.06
PGII within 50% and anti-*H. pylori* IgG within 50%	166	585	1.70	1.15	2.51
PGII within 50% and anti-*H. pylori* IgG increased ≥ 50%	103	274	2.08	1.36	3.19
PGII increased 50% and anti-*H. pylori* IgG decreased ≥ 50%	18	109	1.01	0.57	1.81
PGII increased 50% and anti-*H. pylori* IgG within 50%	72	224	1.87	1.21	2.90
PGII increased 50% and anti-*H. pylori* IgG increased ≥ 50%	85	156	3.18	2.05	4.93

[a] Defined as: (follow-up – baseline)/baseline x 100%

[b] Estimated using generalized estimating equations (GEE) for correlated binary outcomes

Abbreviations: *H. pylori*: *Helicobacter pylori*; PG: pepsinogen

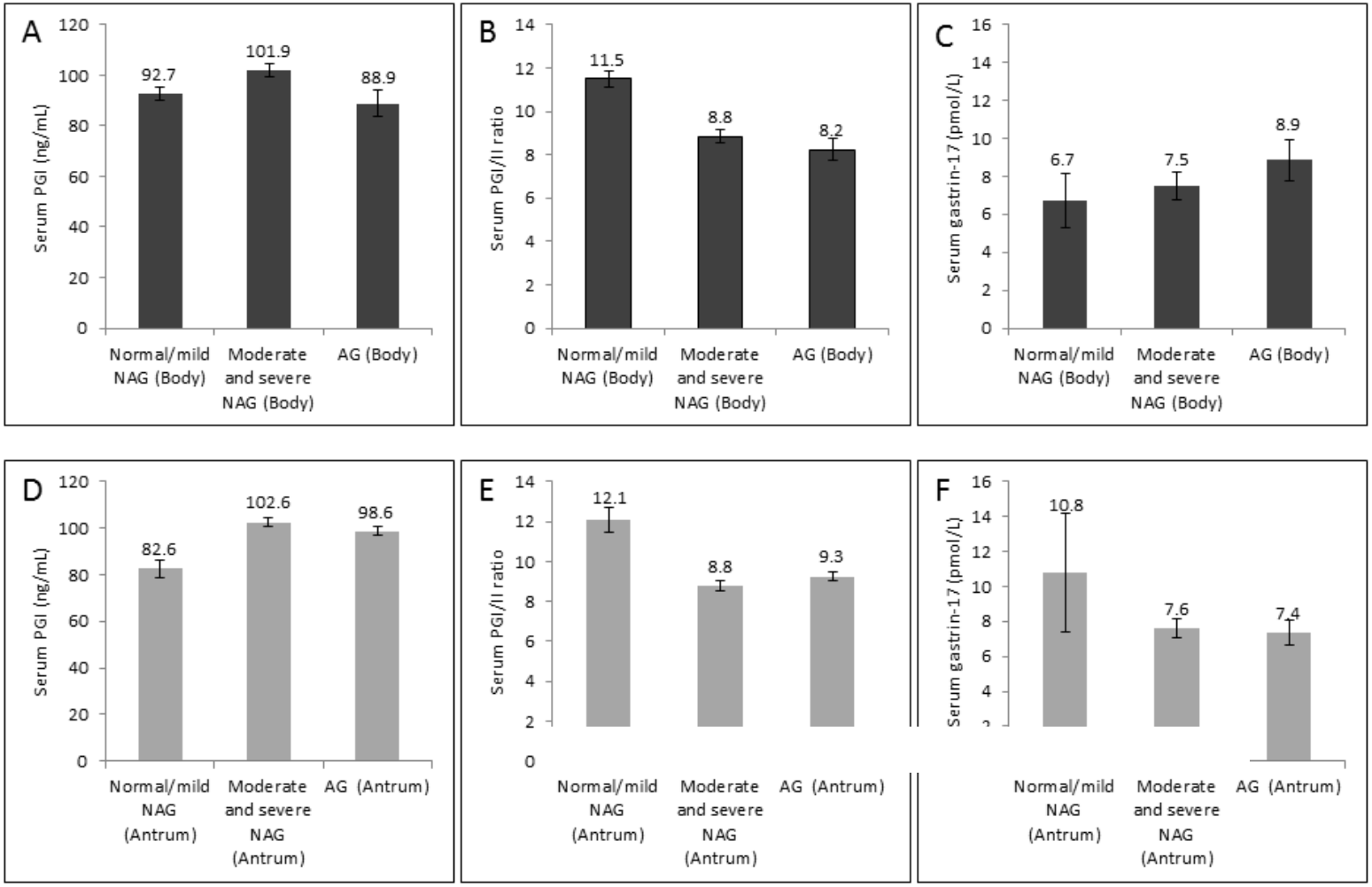

Figure 4.1. Serum pepsinogen I (PGI), PGI/II ratio, and gastrin-17 levels in persons with different gastric histopathologies in the Zhuanghe Gastric Diseases Screening Program, China. (A). Serum PGI levels in persons with different histopathologies in the gastric body; (B). Serum PGI/II ratio in persons with different histopathologies in the gastric body; (C). Serum gastrin-17 levels in persons with different gastric histopathologies in the gastric body; (D). Serum PGI levels in persons with different histopathologies in the gastric antrum; (E). Serum PGI/II ratio in persons with different histopathologies in the gastric antrum; (F). Serum gastrin-17 levels in persons with different histopathologies in the gastric antrum.
Abbreviations: NAG, non-atrophic gastritis; AG, atrophic gastritis.

Supplementary Table 4.1. Associations of relative temporal changes in serum PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG, and gastrin-17 levels with progression of gastric precancerous lesions controlling for age and sex, Zhuanghe Gastric Diseases Screening Program, China.

Relative change [a]	Progression (n)	No progression (n)	OR [b]	95% CI	*P* for trend
Serum PGI					
Decreased ≥ 50%	97	402	1.00	N/A	
Decreased 20 - 50%	148	637	0.96	0.72 1.29	
Within 20%	195	678	1.19	0.90 1.58	< 0.01
Increased 20 - 50%	101	303	1.48	1.07 2.03	
Increased ≥ 50%	123	323	1.62	1.19 2.22	
Serum PGII					
Decreased ≥ 50%	114	589	1.00	N/A	
Decreased 20 - 50%	135	442	1.51	1.16 1.97	
Within 20%	147	503	1.50	1.14 1.96	< 0.01
Increased 20 - 50%	64	242	1.35	0.97 1.88	
Increased ≥ 50%	200	561	1.81	1.40 2.34	
Serum PGI/II ratio					
Increased ≥ 50%	171	698	1.00	N/A	
Increased 20 - 50%	76	254	1.19	0.89 1.61	
Within 20%	148	503	1.19	0.94 1.51	0.05
Decreased 20 - 50%	111	454	0.98	0.76 1.28	
Decreased ≥ 50%	152	409	1.43	1.11 1.85	
Serum gastrin-17					
Decreased ≥ 100%	86	319	1.00	N/A	
Decreased 20 to 100%	106	378	0.95	0.69 1.33	
Within 20%	150	697	0.83	0.61 1.13	0.02
Increased 20 - 500%	162	501	1.15	0.85 1.55	
Increased ≥ 500%	112	309	1.33	0.96 1.84	
Serum anti-*H. pylori* IgG					
Decreased ≥ 50%	114	577	1.00	N/A	

Decreased 20 - 50%	83	367	1.21	0.90	1.62	
Within 20%	140	453	1.55	1.17	2.05	< 0.01
Increased 20 - 50%	63	197	1.61	1.13	2.29	
Increased ≥ 50%	206	528	1.96	1.50	2.55	

[a] Defined as: (follow-up – baseline)/baseline x 100%
[b] Estimated using generalized estimating equations (GEE) for correlated binary outcomes
Abbreviations: *H. pylori*: *Helicobacter pylori*; PG: pepsinogen

Supplementary Table 4.2. Associations of relative temporal changes in serum PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG, and gastrin-17 levels with progression of gastric precancerous lesions using a four-category score system for histopathological diagnosis, Zhuanghe Gastric Diseases Screening Program, China.

Relative change [a]	Progression (n)	No progression (n)	OR [b]	95% CI		*P* for trend
Serum PGI						
Decreased ≥ 50%	70	429	1.00	N/A		< 0.01
Decreased 20 - 50%	103	682	0.98	0.70	1.38	
Within 20%	127	746	1.10	0.79	1.54	
Increased 20 - 50%	67	337	1.30	0.90	1.89	
Increased ≥ 50%	92	354	1.71	1.20	2.43	
Serum PGII						
Decreased ≥ 50%	83	620	1.00	N/A		0.00
Decreased 20 - 50%	92	485	1.37	1.00	1.87	
Within 20%	98	552	1.33	0.97	1.83	
Increased 20 - 50%	38	268	1.03	0.69	1.54	
Increased ≥ 50%	147	614	1.73	1.28	2.35	
Serum PGI/II ratio						
Increased ≥ 50%	120	749	1.00	N/A		0.13
Increased 20 - 50%	55	275	1.28	0.92	1.79	
Within 20%	98	553	1.16	0.87	1.54	
Decreased 20 - 50%	70	495	0.90	0.66	1.24	
Decreased ≥ 50%	114	447	1.45	1.08	1.95	
Serum gastrin-17						
Decreased ≥ 100%	55	350	1.00	N/A		< 0.01
Decreased 20 to 100%	73	411	1.06	0.71	1.58	
Within 20%	108	739	1.03	0.71	1.49	
Increased 20 - 500%	107	556	1.23	0.86	1.75	
Increased ≥ 500%	84	337	1.59	1.09	2.33	
Serum anti-*H. pylori* IgG						
Decreased ≥ 50%	82	613	1.00	N/A		< 0.01
Decreased 20 - 50%	52	398	1.14	0.82	1.60	

Within 20%	101	492	1.64	1.19	2.26
Increased 20 - 50%	44	216	1.63	1.09	2.43
Increased ≥ 50%	138	592	1.78	1.30	2.43

[a] Defined as: (follow-up – baseline)/baseline x 100%
[b] Estimated using generalized estimating equations (GEE) for correlated binary outcomes
Abbreviations: *H. pylori*: *Helicobacter pylori*; PG: pepsinogen

Supplementary Table 4.3. Associations of absolute temporal changes in serum PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG, and gastrin-17 levels with progression of gastric precancerous lesions, Zhuanghe Gastric Diseases Screening Program, China.

Absolute change [a]	Progression (n)	No progression (n)	OR [b]	95% CI		*P* for trend
Serum PGI						
Decreased ≥ 50 ng/mL	107	450	1.00	N/A		< 0.01
Decreased 20 - 50 ng/mL	118	517	0.97	0.71	1.31	
Within 20 ng/mL	255	847	1.24	0.95	1.61	
Increased 20 - 50 ng/mL	115	307	1.69	1.24	2.29	
Increased ≥ 50 ng/mL	69	222	1.37	0.97	1.93	
Serum PGII						
Decreased ≥ 7.5 ng/mL	117	598	1.00	N/A		< 0.01
Decreased 3 - 7.5 ng/mL	117	340	1.69	1.27	2.25	
Within 3 ng/mL	191	688	1.39	1.07	1.81	
Increased 3 - 7.5 ng/mL	91	323	1.49	1.11	2.02	
Increased ≥ 7.5 ng/mL	144	388	1.82	1.37	2.41	
Serum PGI/II ratio						
Increased ≥ 5 units	112	475	1.00	N/A		0.03
Increased 2 - 5 units	96	334	1.22	0.91	1.63	
Within 2 units	211	756	1.18	0.92	1.52	
Decreased 2 - 5 units	94	364	1.08	0.80	1.45	
Decreased ≥ 5 units	145	389	1.48	1.11	1.96	
Serum gastrin-17						
Decreased ≥ 2 pmol/L	111	358	1.00	N/A		0.02
Decreased 0.4 - 2 pmol/L	82	329	0.87	0.62	1.21	
Within 0.4 pmol/L	149	734	0.71	0.53	0.95	
Increased 0.4 - 4 pmol/L	136	431	1.05	0.78	1.41	
Increased ≥ 4 pmol/L	138	352	1.28	0.95	1.72	
Serum anti-*H. pylori* IgG						
Decreased ≥ 20 EIU	113	548	1.00	N/A		< 0.01
Decreased 8 - 20 EIU	76	340	1.04	0.77	1.42	

Within 8 EIU	176	605	1.35	1.03	1.76
Increased 8 - 20 EIU	90	254	1.64	1.20	2.25
Increased ≥ 20 EIU	151	375	1.85	1.38	2.47

[a] Defined as: (follow-up – baseline)
[b] Estimated using generalized estimating equations (GEE) for correlated binary outcomes
Abbreviations: *H. pylori*: *Helicobacter pylori*; PG: pepsinogen

Supplementary Table 4.4. Associations of rate of temporal changes in serum PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG, and gastrin-17 levels with progression of gastric precancerous lesions, Zhuanghe Gastric Diseases Screening Program, China.

Rate of change [a] (per year)	Progression (n)	No progression (n)	OR [b]	95% CI		*P* for trend
Serum PGI level						
Decreased ≥ 20 ng/mL	142	687	1.00	N/A		< 0.01
Decreased by 5 - 20 ng/mL	137	490	1.12	0.86	1.45	
Within 5 ng/mL	137	446	1.13	0.84	1.50	
Increased by 5 - 20 ng/mL	142	355	1.74	1.34	2.28	
Increased by ≥ 20 ng/mL	106	365	1.41	1.07	1.86	
Serum PGII level						
Decreased by ≥ 5 ng/mL	105	553	1.00	N/A		< 0.01
Decreased by 1 - 5 ng/mL	148	499	1.33	1.00	1.77	
Within 1 ng/mL	145	463	1.38	1.02	1.86	
Increased by 1 - 5 ng/mL	136	457	1.41	1.06	1.88	
Increased by ≥ 5 ng/mL	126	365	1.70	1.27	2.28	
Serum PGI/II ratio						
Increased by ≥ 2 units	155	634	1.00	N/A		0.06
Increased by 0.5 - 2 units	92	357	0.94	0.72	1.24	
Within 0.5 units	126	401	1.05	0.80	1.38	
Decreased by 0.5 - 2 units	110	391	1.01	0.78	1.32	
Decreased by ≥ 2 units	175	535	1.27	0.99	1.62	
Serum gastrin-17 level						
Decreased by ≥ 1 pmol/L	111	399	1.00	N/A		< 0.01
Decreased by 0.01 - 1 pmol/L	102	367	0.90	0.66	1.21	
Within 0.01 pmol/L	108	570	0.69	0.51	0.94	
Increased by 0.01 - 1 pmol/L	102	307	1.01	0.74	1.38	
Increased by ≥ 1 pmol/L	193	561	1.20	0.92	1.58	
Serum anti-*H. pylori* IgG level						
Decreased by ≥ 10 EIU	122	596	1.00	N/A		0.03
Decreased by 2 - 10 EIU	109	451	1.02	0.77	1.36	

Within 2 EIU	96	314	1.24	0.91	1.68
Increased by 2 - 10 EIU	121	349	1.41	1.05	1.89
Increased by ≥ 10 EIU	158	412	1.78	1.35	2.34

[a] Defined as: (follow-up – baseline)/follow-up time interval
[b] Estimated using generalized estimating equations (GEE) for correlated binary outcomes
Abbreviations: *H. pylori*: *Helicobacter pylori*; PG: pepsinogen

Supplementary Table 4.5. Associations of relative temporal changes in serum PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG, and gastrin-17 levels with progression of gastric precancerous lesions when the follow-up visit was compared with the previous visit, Zhuanghe Gastric Diseases Screening Program, China.

Relative change [a]	Progression (n)	No progression (n)	OR [b]	95% CI		*P* for trend
Serum PGI						
Decreased ≥ 50%	92	348	1.00	N/A		
Decreased 20 - 50%	149	592	0.92	0.69	1.21	
Within 20%	199	650	1.16	0.89	1.51	< 0.01
Increased 20 - 50%	105	311	1.27	0.93	1.73	
Increased ≥ 50%	163	384	1.56	1.16	2.11	
Serum PGII						
Decreased ≥ 50%	122	556	1.00	N/A		
Decreased 20 - 50%	136	431	1.44	1.10	1.88	
Within 20%	156	499	1.44	1.11	1.86	< 0.01
Increased 20 - 50%	66	266	1.12	0.81	1.55	
Increased ≥ 50%	224	530	1.92	1.49	2.46	
Serum PGI/II ratio						
Increased ≥ 50%	205	737	1.00	N/A		
Increased 20 - 50%	80	246	1.21	0.91	1.62	
Within 20%	141	477	1.09	0.86	1.39	0.01
Decreased 20 - 50%	120	410	1.08	0.84	1.39	
Decreased ≥ 50%	154	381	1.50	1.17	1.92	
Serum gastrin-17						
Decreased ≥ 100%	117	411	1.00	N/A		
Decreased 20 to 100%	92	302	1.09	0.81	1.47	
Within 20%	160	651	0.82	0.64	1.06	0.08
Increased 20 - 500%	174	461	1.31	1.01	1.70	
Increased ≥ 500%	100	291	1.20	0.88	1.61	
Serum anti-*H. pylori* IgG						
Decreased ≥ 50%	120	503	1.00	N/A		
Decreased 20 - 50%	94	324	1.20	0.90	1.61	< 0.01

Within 20%	144	443	1.35	1.04	1.74
Increased 20 - 50%	66	207	1.26	0.91	1.76
Increased ≥ 50%	229	557	1.67	1.30	2.14

[a] Defined as: (follow-up – baseline)/baseline x 100%
[b] Estimated using generalized estimating equations (GEE) for correlated binary outcomes
Abbreviations: *H. pylori*: *Helicobacter pylori*; PG: pepsinogen

Supplementary Table 4.6. Associations of relative temporal changes in serum PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG, and gastrin-17 levels with progression of gastric precancerous lesions when only second visit and baseline visit were included, Zhuanghe Gastric Diseases Screening Program, China.

Relative change [a]	Progression (n)	No progression (n)	OR	95% CI		*P* value	*P* for trend
Serum PGI							
Decreased ≥ 50%	68	283	1.00	N/A		N/A	
Decreased 20 - 50%	95	428	0.91	0.64	1.29	0.60	
Within 20%	120	453	1.09	0.78	1.52	0.63	< 0.01
Increased 20 - 50%	76	205	1.52	1.04	2.20	0.03	
Increased ≥ 50%	84	206	1.66	1.15	2.40	0.01	
Serum PGII							
Decreased ≥ 50%	84	439	1.00	N/A		N/A	
Decreased 20 - 50%	88	310	1.48	1.06	2.06	0.02	
Within 20%	93	334	1.41	1.02	1.97	0.04	< 0.01
Increased 20 - 50%	48	169	1.51	1.02	2.25	0.04	
Increased ≥ 50%	128	320	2.09	1.53	2.86	<.0001	
Serum PGI/II ratio							
Increased ≥ 50%	131	525	1.00	N/A		N/A	
Increased 20 - 50%	51	176	1.16	0.80	1.67	0.44	
Within 20%	90	332	1.09	0.80	1.47	0.59	0.03
Decreased 20 - 50%	75	282	1.07	0.77	1.47	0.69	
Decreased ≥ 50%	92	240	1.55	1.14	2.11	0.01	
Serum gastrin-17							
Decreased ≥ 100%	54	206	1.00	N/A		N/A	
Decreased 20 to 100%	78	297	0.98	0.66	1.45	0.93	
Within 20%	86	392	0.85	0.58	1.24	0.40	0.07
Increased 20 - 500%	122	357	1.32	0.91	1.89	0.14	
Increased ≥ 500%	62	197	1.20	0.79	1.82	0.38	
Serum anti-*H. pylori* IgG							
Decreased ≥ 50%	76	377	1.00	N/A		N/A	
Decreased 20 - 50%	59	248	1.17	0.81	1.71	0.40	< 0.01

Within 20%	96	302	1.53	1.09	2.15	0.01
Increased 20 - 50%	44	140	1.51	0.99	2.30	0.06
Increased ≥ 50%	135	354	1.91	1.39	2.63	<.0001

[a] Defined as: (follow-up – baseline)/baseline x 100%
[b] Estimated using generalized estimating equations (GEE) for correlated binary outcomes
Abbreviations: *H. pylori*: *Helicobacter pylori*; PG: pepsinogen

Supplementary Table 4.7. Associations of relative temporal changes in serum PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG, and gastrin-17 levels with progression of gastric precancerous lesions when only last visit and baseline visit were included, Zhuanghe Gastric Diseases Screening Program, China.

Relative change [a]	Progression (n)	No progression (n)	OR [b]	95% CI		*P* for trend
Serum PGI						
Decreased ≥ 50%	56	245	1.00	N/A		
Decreased 20 - 50%	107	441	1.06	0.74	1.51	
Within 20%	142	469	1.32	0.93	1.87	< 0.01
Increased 20 - 50%	63	192	1.43	0.95	2.15	
Increased ≥ 50%	89	229	1.69	1.15	2.47	
Serum PGII						
Decreased ≥ 50%	77	424	1.00	N/A		
Decreased 20 - 50%	93	306	1.66	1.19	2.33	
Within 20%	108	329	1.79	1.29	2.48	< 0.01
Increased 20 - 50%	41	152	1.49	0.98	2.27	
Increased ≥ 50%	136	356	2.10	1.54	2.88	
Serum PGI/II ratio						
Increased ≥ 50%	119	506	1.00	N/A		
Increased 20 - 50%	51	181	1.19	0.82	1.72	
Within 20%	105	340	1.31	0.97	1.76	< 0.01
Decreased 20 - 50%	76	289	1.11	0.80	1.53	
Decreased ≥ 50%	103	247	1.79	1.32	2.43	
Serum gastrin-17						
Decreased ≥ 100%	69	208	1.00	N/A		
Decreased 20 to 100%	84	320	0.78	0.54	1.12	
Within 20%	82	385	0.64	0.45	0.92	0.27
Increased 20 - 500%	117	384	0.92	0.65	1.29	
Increased ≥ 500%	73	193	1.15	0.78	1.68	
Serum anti-*H. pylori* IgG						

Decreased ≥ 50%	80	375	1.00		N/A	
Decreased 20 - 50%	55	223	1.16	0.79	1.69	
Within 20%	83	306	1.26	0.90	1.78	< 0.01
Increased 20 - 50%	46	140	1.52	1.01	2.30	
Increased ≥ 50%	155	399	1.83	1.35	2.49	

[a] Defined as: (follow-up – baseline)/baseline x 100%
[b] Estimated using generalized estimating equations (GEE) for correlated binary outcomes
Abbreviations: *H. pylori*: *Helicobacter pylori*; PG: pepsinogen

Supplementary Table 4.8. Selected sociodemographic characteristics, risk behaviors, serum biomarker levels and pathological diagnosis of participants of the Zhuanghe Gastric Cancer Study, China.

Characteristics [a]	All participants (n=18,760)	Those included in analysis (n=2,039)
Male (%)	42.7	46.8
Age (yrs.)	49.9 ± 10.8	49.8 ± 10.5
Year at enrollment (%)		
1997 – 1999	21.5	68.1
2002 – 2004	40.4	9.7
2009 – 2011	38.1	22.3
Current smoker (%)		
Yes	22.9	28.7
No	66.1	63.3
Missing	11.0	8.0
Drink alcohol (%)		
Yes	15.3	20.5
No	73.8	71.5
Missing	11.0	8.0
Family history of GC (%)		
Yes	12.1	19.1
No	77.2	72.9
Missing	10.7	19.1
***H. pylori* seropositivity (%)**		
Postive	42.4	48.2
Negative	57.6	51.8
Serum biomarkers		
PGI	95.3 ± 47.4	101.5 ± 53.6
PGII	12.7 ± 11.1	15.8 ± 12.7
PGI/II ratio	10.7 ± 8.5	9.5 ± 9.5
H. pylori antibody IgG	36.9 ± 33.4	41.3 ± 33.3
Gastrin-17	4.7 ± 11.3	3.8 ± 11.4

[a] Table reports % for categorical variables and the mean ± standard deviation for continuous variables.

Chapter 5. Transforming growth factors and receptor as potential modifiable pre-neoplastic biomarkers of risk for colorectal neoplasms

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Running title: TGFα, TGFβ_1, and TGFβ_1 receptor expression in human colorectal mucosa

Key words: Colorectal neoplasms, biological markers, TGFα, TGFβ_1, and TGFβ receptor II

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Abstract

Increased colorectal epithelial cell proliferation is an early, common event in colorectal carcinogenesis. We conducted a pilot, colonoscopy-based case-control study (n = 48 cases, 147 controls) of incident, sporadic colorectal adenoma to investigate endogenous cell growth factors and receptor, as well as the balance of growth factors, as potential modifiable pre-neoplastic biomarkers of risk for colorectal neoplasms. We measured transforming growth factor alpha (TGFα), TGFβ_1, and TGFβ receptor II (TGFβRII) expression in normal-appearing mucosa from the rectum, sigmoid colon, and ascending colon using automated immunohistochemistry and quantitative image analysis. Diet and lifestyle were assessed via questionnaires. The mean ratio of rectal TGFα to TGFβ_1 expression and mean rectal TGFα expression were, respectively, 110% (P=0.02) and 49% (P=0.04) higher in cases than in controls, and associated with a more than two-fold (OR 2.42, 95% CI 0.85 – 6.87) and a 62% (OR 1.62, 95% CI 0.63 – 4.19) higher risk of colorectal adenoma. TGFβ_1 and TGFβRII expression were 6.7% (P=0.75) and 7.2% (P=0.49), respectively, lower in cases than in controls. The TGF /TGFβ_1 expression ratio was 105% higher among smokers than among non-smokers (P=0.03). These preliminary data suggest that the balance of TGFα and TGFβ_1 expression, and to a lesser extend TGFα alone, in the normal-appearing rectal mucosa may be directly associated with risk for incident, sporadic colorectal neoplasms, as well as with modifiable risk factors for colorectal neoplasms.

Introduction

Despite advances in screening and treatment, colorectal cancer remains the second most common cause of cancer death in the United States (362) and the fourth most common cause of cancer death worldwide (1). It is widely accepted that most colorectal carcinomas develop from adenomas (6). Adenomas recur in about 32% of people in three years after removal (497), suggesting that normal-appearing tissue may retain components of risk.

Currently, there are no generally accepted pre-neoplastic biomarkers of risk for colorectal cancer, and normal-appearing tissue is a promising place to begin the search for biomarkers. The most studied candidate has been colorectal epithelial cell proliferation (525). Growth factors stringently regulate cell proliferation (476), and likely contribute to or at least affect colorectal carcinogenesis (478). Transforming growth factor alpha (TGFα) and transforming growth factor beta (TGFβ) are autocrine/paracrine growth factors that are classically thought of as potent promoters and inhibitors of cell growth, respectively, in normal tissues (476, 477). TGFα acts through the EGF receptor, TGFβ acts through the TGFβ receptor (TGFβR), and both receptors are abundantly expressed in the normal colon epithelium (483). TGFβ has three isoforms, including $TGF\beta_1$, $TGF\beta_2$, and $TGF\beta_3$; $TGF\beta_1$ is expressed in epithelial cells (482). The growth inhibitory signal of TGFβ is transduced through two receptors, type I (RI) and type II (RII) (482). TGFβRII inactivation is found in colorectal cancers and related cell lines with microsatellite instability (MSI) (484, 485), and MSI colorectal tumors tend to arise in the proximal colon (485).

We previously reported that TGFα may serve as a modifiable biomarker of risk for colorectal cancer, as expression of this pro-growth marker was higher in the normal-

appearing colorectal mucosa of persons with adenoma (526). Given the complex interaction of factors involved in the development of colorectal neoplasms, it is unlikely that a single biomarker will be sufficient to assess or manage risk. Since our results on TGFα were published, we quantified the expression of two other related proteins, $TGF\beta_1$ and TGFβRII, in the normal-appearing colorectal mucosa of persons at varied risk for colorectal cancer and increased the sample size for TGFα in order to investigate a TGFα/TGFβ1 ratio, an indicator of the balance between the growth-promoting and -inhibiting factors.

To our knowledge, no other study has investigated the quantitative expression of all three cell proliferation/growth-related proteins—TGFα, $TGF\beta_1$, and TGFβRII—in the normal-appearing colorectal mucosa as potential modifiable pre-neoplastic biomarkers of risk for colorectal neoplasms. To address this need, we investigated the expression of these potential markers, individually and combined, in biopsies of normal-appearing colorectal mucosa and assessed their associations with risk for colorectal adenoma as well as with potential risk factors for colorectal neoplasms.

Materials and Methods

Study Population

Rectal biopsy samples were procured from participants in the Markers of Adenomatous Polyps II study (MAP II), a colonoscopy-based, case-control study of incident sporadic colorectal adenomas described in detail elsewhere (526-529). Briefly, the MAP II study was designed to investigate whether the expression patterns of various genes and other factors implicated in colorectal carcinogenesis in normal-appearing rectal mucosa are associated with adenomas and thus could be possible biomarkers of risk for colorectal neoplasms. Adults aged 30-74 years were recruited upon referral for

routine outpatient elective colonoscopy at Consultants in Gastroenterology, PA, a large private practice gastroenterology group in Columbia, SC. Subjects were excluded if they had a history of previous adenomatous polyps, familial adenomatous polyposis, inflammatory bowel disease, colon resection, or any cancer other than non-melanoma skin cancer. Among those eligible for the study, the consent rate was 87.5%.

Data Collection

Prior to undergoing colonoscopy, MAP II participants (n = 203; 49 cases, 154 controls) completed mailed questionnaires eliciting self-reported demographics, medical history, anthropometrics, and lifestyle characteristics; diet was assessed with a Willett Food Frequency Questionnaire (530). Colonoscopies were performed in the usual manner following a 12-hour fast and polyethylene glycol bowel cleansing preparation. All participants had six "pinch" biopsies taken from the normal-appearing rectal mucosa (10 cm above the anus). On 30% of the participants, biopsies from the mid-sigmoid and proximal ascending colon were also collected. No biopsies were taken within 4 cm of a polyp. Five patients were excluded because of missing covariate data, and three were excluded because of implausible self-reported energy intakes (≤ 500 kcal/d or ≥ 5,000 kcal/d). Because of limited funding (and for proximal colon sites, tissues) biomarkers were measured in only randomly selected subsets of participants as follows: $TGF\beta_1$ expression was measured in rectal biopsies from 86 participants (43 cases, 43 controls); TGFβRII was measured in rectal biopsies from 37 participants (18 cases, 19 controls); TGFα expression was measured in sigmoid and ascending colon biopsies in 19 participants (9 cases, 10 controls); and $TGF\beta_1$ expression was evaluated in the sigmoid colon in 57 participants (18 cases, 39 controls) and in the ascending colon in 60 participants (16 cases, 44 controls). We previously reported on the expression of TGFα in the rectum of 60 participants (29 cases, 31 controls) (526), and for the current

analysis we measured TGFα expression in an additional 11 participants (6 cases and 5 controls), for a total of 71 participants (35 cases, 36 controls).

Laboratory Procedures

All biopsy specimens were fixed with 10% normal buffered formalin for 24 hours then transferred to 70% ethanol. Within a week, the specimens were processed and embedded in paraffin blocks with three biopsies per colon site per block. The paraffin blocks were then cut into 3.0 micron-thick sections with each level 40 microns apart. Five slides with four biopsy levels each were processed and stained within seven days of being cut, yielding a total of 20 biopsy levels per biomarker per colon site per patient. Using automated procedures, the slides underwent immunohistochemical processing (described in detail elsewhere (526)) with TGFα antibody (Neomarkers, Fremont, CA, MS1000P, in a 1:50 dilution), $TGF\beta_1$ (Santa Cruz, Dallas, TX, sc-146, in a 1:75 dilution), or TGFβRII (Santa Cruz, Dallas, TX, sc-1700, in a 1:150 dilution).

Image Analysis

A quantitative image analysis method to measure the expression of biomarkers in normal colorectal crypts was described in detail previously (526-529). Briefly, the image analysis unit was a “hemicrypt”, defined as one side of a colorectal crypt bisected from base to colorectal lumen surface. A “scorable” hemicrypt was defined as an intact hemicrypt extending from the muscularis mucosa to the colorectal lumen. Before image analysis, staining adequacy was checked by examining the negative and positive control slides in each batch. The key equipment and software for the image analysis procedures were: personal computer, light microscope (Olympus BX40, Olympus Corporation, Japan) with appropriate filters and attached digital light microscope camera (Polaroid DMC Digital Light Microscope Camera, Polaroid Corporation, USA), digital

drawing board, ImagePro Plus image analysis software (Media Cybernetics, Inc., MD), our in-house developed plug-in software for colorectal crypt analysis, and Microsoft Access 2003 relational database software (Microsoft Corporation, WA).

Prior to image analysis, the scorer, who was blinded to the case-control status, cleaned the slides, oriented them in a standardized fashion, standardized the equipment and image software light settings, selected the two of the three biopsies with the greatest number of scorable hemicrypts, created background correction images for each slide, and captured 16-bit gray scale images of crypts at 200x magnification. Then, the scorer traced the border of the "hemicrypt" (one half of the crypt) in the image analysis program (**Figure 5.1**). The program then divided the traced hemicrypt into segments corresponding in width to that of an average normal colonocyte, and the background-adjusted optical density of the labeling across the whole hemicrypt and within each segment was measured and exported to a Microsoft Access database along with various dimensional parameters of the hemicrypt. The goal was to score at least 16 to 20 hemicrypts per colorectal site for each biomarker.

Reliability control was performed by having the scorer re-analyze 10% of the slides; the slides were randomly selected and the scorer was blinded to the selection. Intra-reader reliability for each biomarker was >0.90.

Statistical Analysis

Cases included participants with pathology-confirmed, incident, sporadic colorectal adenomas. Participants without adenoma on colonoscopy were considered controls. Baseline characteristics of cases and controls in the entire MAP II study population as well as in the subsets of participants for whom slides were

immunohistochemically processed and analyzed were compared using the Student's t-test for continuous variables and Fisher's exact test for categorical variables.

The primary variable of interest was the biomarker labeling optical density ("biomarker expression") in the crypts of the colorectal mucosa, and the primary analyses of interest were the associations of the biomarkers with adenoma. Biomarker values were standardized for staining batch by taking the value in each individual divided by the mean of the staining batch in which the individual's sample was processed for all analyses. In a sensitivity analysis, staining batch was included in the models described below as a fixed effect covariate.

For graphical analyses, the distributions of batch-standardized TGFα, TGFβ_1, and TGFβRII labeling optical densities along the full lengths of the hemicrypts were plotted and modeled using the LOESS procedure. First, each hemicrypt was standardized to 50 sections; then, the batch-standardized average of each section across all hemicrypts was calculated and predicted by the LOESS model separately for cases and controls; and then the results were graphically plotted along with smoothing lines.

For quantitative analyses, the mean batch-standardized biomarker expression across the hemicrypts from a colon or the rectal site on a patient was calculated by summing the labeling optical densities from all analyzed hemicrypts from the biopsy specimens and dividing by the number of hemicrypts (mean = 29 ± SD 8) analyzed from that site. A TGFα/TGFβ_1 ratio was calculated as an indicator of the balance between the growth-promoting and -inhibiting factors by dividing the mean batch standardized level of TGFα by that of TGFβ_1; a higher ratio, thus, would indicate a more pro-growth balance.

Analysis of covariance (ANACOVA) was used to assess age- and sex-adjusted mean case-control differences in the biomarker variables. Unconditional logistic regression was used to calculate odds ratios (OR) with 95% confidence intervals (95% CI) to assess associations of the biomarkers with colorectal adenoma; for these analyses, batch-standardized biomarker expression was dichotomized based on the median value among the controls.

ANACOVA was used to evaluate mean age- and sex- adjusted differences in batch-standardized biomarker expression in the rectum according to diet and lifestyle. For these analyses we dichotomized the risk factor variables and combined the cases and controls.

All statistical analyses were performed using SAS 9.3 statistical software (SAS Institute Inc., Cary, NC). A P value ≤0.05 (two-sided) was considered statistically significant. Because of the limited sample sizes for assessing biomarkers in the sigmoid and ascending colon and the similarity of the findings for these colon sites to those for the rectum, only the results for the rectal mucosa are presented in the tables.

Results

Study population

Selected characteristics of the 43 cases and 43 controls analyzed for $TGF\beta_1$ are presented in **Table 5.1**. Cases tended to be less likely to take nonsteroidal anti-inflammatory drugs (NSAIDs) regularly, more likely to be smokers, and, on average, to have higher total energy intakes than did controls. These baseline characteristics were comparable to those in the full MAP II study population (**Supplementary Table 5.1**) and on whom we measured TGFα or TGFβRII (data not shown).

Case-control differences in biomarker expression

Graphical assessments of TGFβ_1 and TGFβRII distributions along the full lengths of rectal crypts by case-control status are presented in **Figure 5.2**; the graphical assessment for TGFα expression was reported previously (526). Numerical assessments of differences between cases and controls in the expression of all markers and their associations with adenoma are presented in **Table 5.2**.

The ratio of TGFα to TGFβ_1 was 110% higher in cases than controls (p=0.02), and a larger TGFα/TGFβ_1 ratio was associated with a more than two-fold higher risk of adenoma (OR 2.42, 95% CI 0.85 - 6.87).

In the graphical assessment, TGFβ_1 expression in cases and controls along the full lengths of rectal crypts decreased gradually from the crypt base to the apex, and there were no apparent case-control differences (**Figure 5.2, Panel A**). In the numerical assessment, age- and sex-adjusted TGFβ_1 expression was 6.7% lower in cases than controls, but the difference was not statistically significant (p=0.75).

The graphical assessment for TGFα expression was reported previously (526). Briefly, TGFα expression was higher in cases than in controls, and the difference was uniform along the full lengths of rectal crypts. In the numerical assessment (**Table 5.2**), age and sex-adjusted TGFα expression was proportionally 49% higher in cases than in controls (p=0.04), which is similar to the results we reported previously with a smaller sample size (51% higher, p=0.05) (526). Higher TGFα expression was associated with approximately 62% higher risk of adenoma (OR 1.62, 95% CI 0.63 - 4.19).

In the graphical assessment, TGFβRII expression in cases and controls along the full lengths of rectal crypts was relatively uniform from the crypt base to the apex,

and there were no apparent case-control differences (**Figure 5.2, Panel B**). In the numerical assessment, age- and sex-adjusted TGFβRII expression was 7.2% lower in cases than in controls, but the difference was not statistically significant (p=0.49).

Adjustment for batch as a fixed covariate in the models produced results comparable in magnitude and direction to those presented in Table 2 (data not shown). Further adjustments for energy intake, NSAID use, and smoking status (Table 2) or multiple other risk factors (data not shown) did not appreciably change the results.

The findings for TGFα/TGFβ_1 ratio, TGFβ_1, and TGFα for the ascending and sigmoid colon were similar to those for the rectum; however, with the small sample sizes, none of the findings was statistically significant (data not shown).

For each biomarker, we calculated the mean coefficient of variation (CV) across all patients to represent the inter-crypt correlation within an individual. The mean CVs were 69%, 78%, and 23% for TGFα, TGFβ_1, and TGFβRII, respectively, underscoring the importance of evaluating multiple hemicrypts/biomarker/person.

Associations of biomarkers with potential risk factors

In our analyses of potential associations of 20 known and suspected risk factors for colorectal neoplasms with the TGFα/TGFβ_1 ratio, TGFβ_1, TGFα, and TGFβRII (data shown only for differences in the TGFα/TGFβ_1 ratio according to smoking, serum 25(OH)-vitamin D_3, age, and BMI [**Figure 3**]), the only statistically significant finding was that the TGFα/TGFβ_1 ratio was 105% higher among smokers than among non-smokers (p=0.03). TGFβ_1 expression was 52.2% lower (p=0.06) among smokers than among non-smokers. Consistent with findings from our randomized controlled trial of the effects of supplemental calcium and vitamin D_3 on normal rectal tissue biomarkers of risk for

colorectal neoplasms (531), the TGFα/TGFβ_1 ratio was 44.7% lower in those with higher serum 25(OH)-vitamin D_3 levels and 16.4% lower in those with higher total calcium intakes, but these findings were not statistically significant. Otherwise, no consistent patterns of associations of the risk factors with any of the biomarkers were noted.

Discussion

Our preliminary data suggest that the balance of TGFα and TGFβ_1 expression, and, to a lesser extent, TGFα alone in the normal rectal mucosa may be directly associated with risk for incident, sporadic colorectal neoplasms, providing support for further investigation of these growth factors as potential biomarkers of risk for colorectal neoplasms. Our data are also consistent with weaker inverse associations of TGFβ_1 and TGFβRII with adenomas.

Consistent with our finding that TGFα expression was greater in the normal-appearing colorectal mucosa of incident, sporadic colorectal adenoma patients than in adenoma-free individuals, other studies found that TGFα expression was greater in colorectal adenomas and cancers (532, 533), and in the blood of colorectal cancer patients (534, 535). A dual role of TGFβ_1 has been proposed, because TGFβ_1 suppresses the growth of normal epithelial cells but promotes tumor metastasis in later stages of cancer (477, 482, 536, 537). In a mouse model, the absence of TGFβ_1 expression promoted the progression from hyperplasia to adenoma and allowed the development of carcinoma (486). In colorectal adenomas (538-540) and cancers (538-543) TGFβ_1 was found to be overexpressed and directly associated with more advanced tumor characteristics.

The mechanism(s) linking higher TGFα expression and, especially, a more pro-growth balance of TGFα and TGFβ_1 expression with an increased risk of colorectal

neoplasms is unclear. Cell proliferation is regulated by growth factors (476), and TGFα and TGFβ are two classes of polypeptide growth factors. Numerous studies reported that, compared to patients at low risk for colon cancer, patients with colon cancer and patients in every category known to be at higher risk for colon cancer, on average, exhibit in their normal-appearing mucosa both an increased colonic epithelial cell proliferation rate and an extension of the colon crypt proliferative zone from the lower (basal) 60% of the crypt to include the upper (luminal) 40% of the crypt (525). In patients with previous colon cancer or sporadic adenomas, these changes also predicted adenoma recurrence (544, 545).

TGFα, a potent stimulator of colonocyte growth/proliferation (546), can synergize with c-myc to promote malignant transformation *in vitro* (483), and $TGF\beta_1$, a potent inhibitor of colonocyte growth/proliferation, inhibits c-myc (483, 547) and induces p21 (547) and cyclin-dependent kinase inhibitors (548). High TGFα expression and, especially, a more pro-growth balance of TGFα and $TGF\beta_1$ expression may increase the number of dividing cells in the colorectal crypt, and DNA is more susceptible to damage during cell division. Therefore, higher TGFα expression and, especially, a more pro-growth balance of TGFα to $TGF\beta_1$ expression may be associated with higher risk of colorectal neoplasms by increasing colorectal epithelial cell proliferation.

We investigated TGFβRII expression in the normal colorectal mucosa because approximately 15% of sporadic colon cancers arise via epigenetic silencing of one or more mismatch repair genes (predominately MSH2 and MLH1), which leads to inactivating mutations of the *TGFβRII* and *bax* genes (549, 550). We previously reported from the same study reported herein that both MSH2 and MLH1 expression in adenoma cases relative to the controls was statistically significantly 49% lower in the ascending colon and, respectively, 23% ($p = 0.06$) and 9% ($p = 0.18$) lower in the rectum

(527, 528). In the present study we observed only modestly less (7 – 8%) TGFβRII expression in the normal-appearing rectal mucosa of the adenoma cases relative to the controls; however, with our small sample size for this analysis, the estimated difference was not statistically significant and we were unable to measure TGFβRII expression in more proximal areas of the colon.

Little is known about whether plausible risk factors for colorectal cancer modify cell growth signaling in the colorectum; therefore, we investigated whether the expression of the TGFα/TGFβ_1 ratio, TGFβ_1, TGFα, and TGFβRII in normal-appearing rectal tissue differed across categories of plausible risk factors for colorectal neoplasms. We found that smokers had a substantial, statistically significant higher level of the TGFα/TGFβ_1 ratio, indicating a more pro-growth balance. On the one hand, given the multiple comparisons involved in this set of analyses, the result should be interpreted cautiously. On the other hand, smoking is significantly associated with increased risk for colorectal cancer (379), and nicotine, one of the major components of cigarette smoke, can stimulate proliferation of colon cancer cells through epidermal growth factor receptor mediated pathways (380-382). Furthermore, consistent with our result that smokers had lower levels of TGFβ_1 expression in their normal-appearing rectal tissue, a study of bone fracture patients found smoking to be associated with lower serum TGFβ_1 concentrations (551). Our preliminary findings also suggest that the TGFα/TGFβ_1 ratio may vary according to other modifiable risk factors (e.g., calcium intake and serum 25(OH)D_3, consistent with the findings of our randomized, controlled calcium and vitamin D_3 trial (531)), although, with our small sample, these differences were not statistically significant.

Our study had several limitations. First, it was a pilot study with a limited sample size, especially for our analyses of TGFβRII and those involving more proximal levels of

the colon and associations of the growth factors with various risk factors. However, in our primary analyses we found statistically significant case-control differences in the TGFα/TGFβ_1 ratio and TGFα even after controlling for multiple potential confounders. Second, despite much of our immunohistochemical procedures being automated, batch variability inevitably is a source of measurement error; however, different ways of controlling for batch variability produced similar results, and these ways were previously shown to sufficiently control for batch variability (552). Finally, temporality could not be established in our study, and larger prospective studies are warranted to investigate whether the TGFα/TGFβ_1 ratio and TGFα predict the occurrence of colorectal neoplasms.

The strengths of our study are: (i) to our knowledge, it is the first to quantify and characterize the expression of the TGFα/ TGFβ_1 ratio, TGFβ_1, and TGFβRII in the normal-appearing colorectal mucosa of incident, sporadic colorectal adenoma patients and adenoma-free individuals; and (ii) the automated immunostaining and image analysis software that allowed quantification of biomarker expression overall as well as their distributions within the colorectal crypts; (iii) the high biomarker measurement reliability; (iv) assignment of case-control status by colonoscopy and measurement of exposure variables prior to colonoscopy to reduce outcome misclassification and reporting bias.

In summary, there is a clear need for treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms, and the results from our pilot study suggest that the balance of TGFα and TGFβ_1 expression and TGFα expression alone in the normal rectal mucosa are promising candidate markers. Our data are also consistent with weaker inverse associations of TGFβ_1 and TGFβRII with adenomas. The findings from the present study, together with our previous findings, suggest that molecular phenotypic differences

in the normal-appearing colorectal mucosa may be associated with increased risk of colorectal neoplasms (526-529) and are modifiable (531, 553-559), providing support for larger, prospective investigations of molecular phenotypic markers as potential modifiable biomarkers of risk for colorectal neoplasms.

Authors' Contributions:

Conception and design: RM Bostick

Development of methodology: RM Bostick

Acquisition of data: CR Daniel, AG Gonzalez-Feliciano, ME Seabrook

Analysis and interpretation of data: H Tu, RM Bostick, TU Ahearn

Writing, review, and/or revision of the manuscript: H Tu, RM Bostick

Administrative, technical, or material support: RM Bostick

Study supervision: RM Bostick

Figure legends

Figure 5.1. Quantitative image analysis process. A, identifying scorable crypts; B, tracing hemicrypt; C, automated sectioning of the trace; and D, automated quantification TGFβ_1 labeling optical density in the whole hemicrypt and each section.

Figure 5.2. Batch-standardized expression (as optical density of biomarker labeling) of (A) TGFβ_1 and (B) TGFβRII along lengths of normal rectal crypts by case-control status. **Abbreviations**: TGFβ_1, transforming growth factor beta 1; TGFβRII, TGFβ receptor II

Figure 5.3. TGFα/TGFβ_1 expression ratio in biopsies of normal-looking rectal mucosa according to selected risk factors for colorectal neoplasms. **Abbreviations**: TGFα, transforming growth factor alpha; TGFβ_1, transforming growth factor beta 1; BMI, body mass index

Table 5.1. Selected characteristics of incident, sporadic colorectal adenoma cases and healthy controls with rectal biopsy $TGF\beta_1$ measurements

Characteristics [a]	Cases (n = 43)	Controls (n = 43)	P-value [b]
Demographics, medical history, habits, anthropometrics			
Age (yrs.)	56.0 ± 6.6	56.1 ± 8.5	0.94
Male (%)	51.2	55.8	0.83
Caucasian (%)	97.7	97.7	1.00
1° relative with colorectal cancer (%)	12.2	19.5	0.55
Take NSAID ≥ 1 week (%)	34.9	41.9	0.66
Take aspirin ≥ 1 week (%)	37.2	41.9	0.83
Use HRT (females) (%)	89.5	77.8	0.40
Current smoker (%)	25.6	4.7 [c]	0.01
Consume alcohol currently (%)	65.1	62.8	1.00
Body mass index (kg/m^2)	31.1 ± 7.4	31.1 ± 7.2	0.99
Waist-to-hip ratio	0.93 ± 0.10	0.94 ± 0.15	0.95
Moderate/vigorous physical activity (METs/day)	26.2 ± 19.1	24.3 ± 17.9	0.63
Serum 25(OH)-vitamin D_3, ng/mL	27.2 ± 12.3	28.6 ± 12.2	0.63
Dietary intakes [d]			
Total energy (kcal/day)	1,957 ± 763	1,565 ± 421	< 0.01
Total fat (g/day)	66.6 ± 16.4	67.0 ± 14.9	0.88
Saturated fat (g/day)	22.0 ± 5.9	22.1 ± 5.2	0.92
Total folate (μg/day) [e]	463.6 ± 224.2	541.0 ± 284.1	0.16
Total fiber (g/day) [e]	15.0 ± 5.5	15.6 ± 6.0	0.61
Total calcium (mg/day) [e]	918.8 ± 495.8	988.6 ± 495.6	0.52
Total vitamin D (IU/day) [e]	327.4 ± 291.3	385.3 ± 286.7	0.36
Fruits and vegetables (servings/wk.)	29.5 ± 16.0	25.0 ± 11.2	0.13
Red meat (servings/wk.)	6.1 ± 4.0	5.6 ± 5.7	0.61
Processed meats (servings/wk.)	2.8 ± 2.6	2.4 ± 1.8	0.34

[a] Table reports % for categorical variables and mean ± standard deviation for continuous variables

[b] From Fisher's exact test for categorical variables and t test for continuous variables

[c] Among all controls (n = 154) was 14.3%

[d] Energy adjusted using residual method

[e] Total values include diet and supplements

Abbreviations: CRC, colorectal cancer; HRT, hormone replacement therapy; NSAID, nonsteroidal anti-inflammatory drug; MET, metabolic equivalents

Table 5.2. TGFβ_1, TGFα, TGFβRII, and TGFα/TGFβ_1 expression ratio in normal-appearing rectal mucosa, by case-control status [a]

Marker	Mean	SE	Mean	SE	% diff [b]	P-value [c]	OR (95% CI)	Model covariates
TGFβ_1	**Cases (N = 43)**		**Controls (N = 43)**					
	0.99	0.16	1.06	0.16	-6.7	0.75	0.83 (0.35 - 1.95)	Age and sex
	0.92	0.21	1.02	0.19	-9.3	0.69	0.93 (0.38 - 2.24)	Age, sex, and family hx CRC
	0.99	0.16	1.06	0.17	-6.3	0.78	0.80 (0.32 - 1.98)	Age, sex, and energy intake
	0.99	0.16	1.06	0.16	-6.5	0.76	0.84 (0.35 - 1.97)	Age, sex, and NSAID use
	0.84	0.18	0.79	0.22	6.7	0.82	1.09 (0.44 - 2.72)	Age, sex, and smoking
TGFα	**Cases (N = 35)**		**Controls (N = 36)**					
	1.19	0.13	0.80	0.13	49.3	0.04	1.62 (0.63 - 4.19)	Age and sex
	1.29	0.17	0.84	0.15	52.7	0.03	1.88 (0.68 - 5.18)	Age, sex, and family hx CRC
	1.23	0.14	0.75	0.13	64.2	0.02	1.91 (0.69 - 5.27)	Age, sex, and energy intake
	1.21	0.14	0.79	0.13	53.3	0.03	1.90 (0.70 - 5.13)	Age, sex, and NSAID use
	1.15	0.15	0.74	0.17	55.4	0.04	1.64 (0.62 - 4.33)	Age, sex, and smoking
TGFβRII	**Cases (N = 18)**		**Controls (N = 19)**					
	0.98	0.08	1.05	0.07	-7.2	0.49	0.83 (0.21 - 3.31)	Age and sex
	0.98	0.11	1.05	0.09	-5.9	0.61	1.04 (0.23 - 4.68)	Age, sex, and family hx CRC
	0.99	0.07	1.05	0.07	-5.0	0.62	1.03 (0.23 - 4.52)	Age, sex, and energy intake
	0.97	0.08	1.06	0.08	-7.7	0.49	0.81 (0.19 - 3.51)	Age, sex, and NSAID use
	0.95	0.08	0.97	0.11	-2.0	0.88	0.66 (0.13 - 3.33)	Age, sex, and smoking
TGFα/TGFβ_1 ratio	**Cases (N = 35)**		**Controls (N = 29)**					
	2.28	0.33	1.09	0.36	109.9	0.02	2.42 (0.85 - 6.87)	Age and sex
	2.40	0.43	1.14	0.42	110.9	0.02	2.54 (0.84 - 7.66)	Age, sex, and family hx CRC
	2.24	0.33	1.14	0.37	97.0	0.04	2.37 (0.79 - 7.11)	Age, sex, and energy intake
	2.45	0.32	1.07	0.35	129.6	0.01	2.44 (0.85 - 7.05)	Age, sex, and NSAID use
	2.60	0.38	1.63	0.49	59.1	0.06	2.44 (0.82 - 7.26)	Age, sex, and smoking

[a] Marker expression quantified by batched-standardized optical density values

[b] % diff = proportional difference, calculated as {(mean in cases- mean in controls) ÷ mean in controls} *100%

[c] p-value for difference by ANACOVA

Abbreviations: SE, standard error; hx CRC, history of colorectal cancer (in a first degree relative); NSAID, nonsteroidal anti-

inflammatory drug (≥ once/wk.)

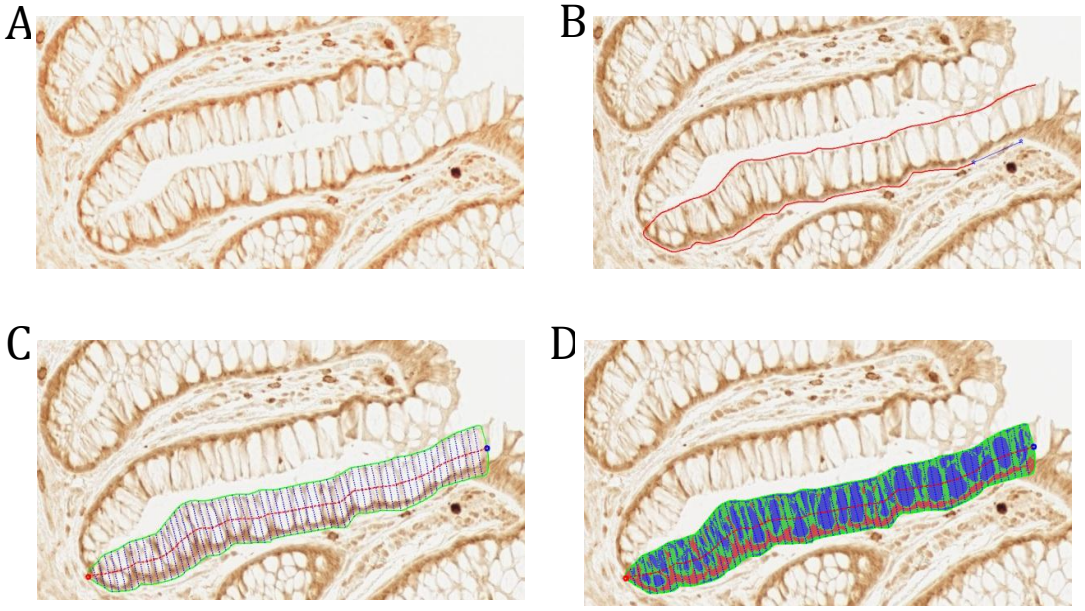
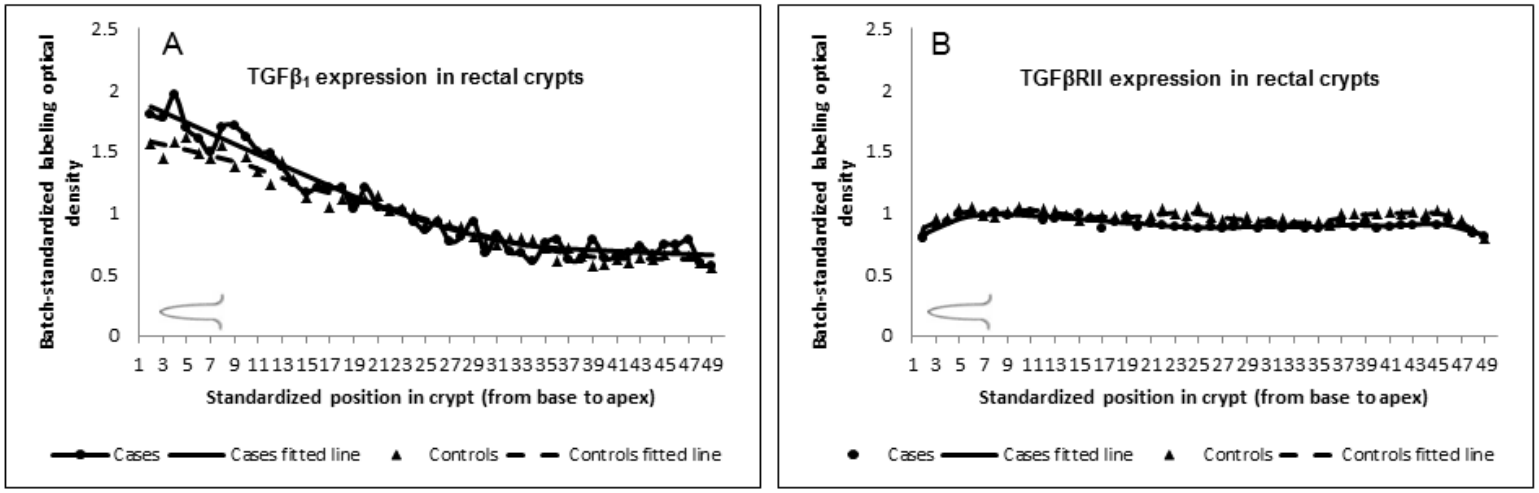


Figure 5.1. Quantitative image analysis process. **A**, identifying scorable crypts; **B**, tracing hemicrypt; **C**, automated sectioning of the trace; and **D**, automated quantification TGFβ_1 labeling optical density in the whole hemicrypt and each section.



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Abbreviations: $TGF\beta_1$, transforming growth factor beta 1; TGFβRII, transforming growth factor beta receptor II

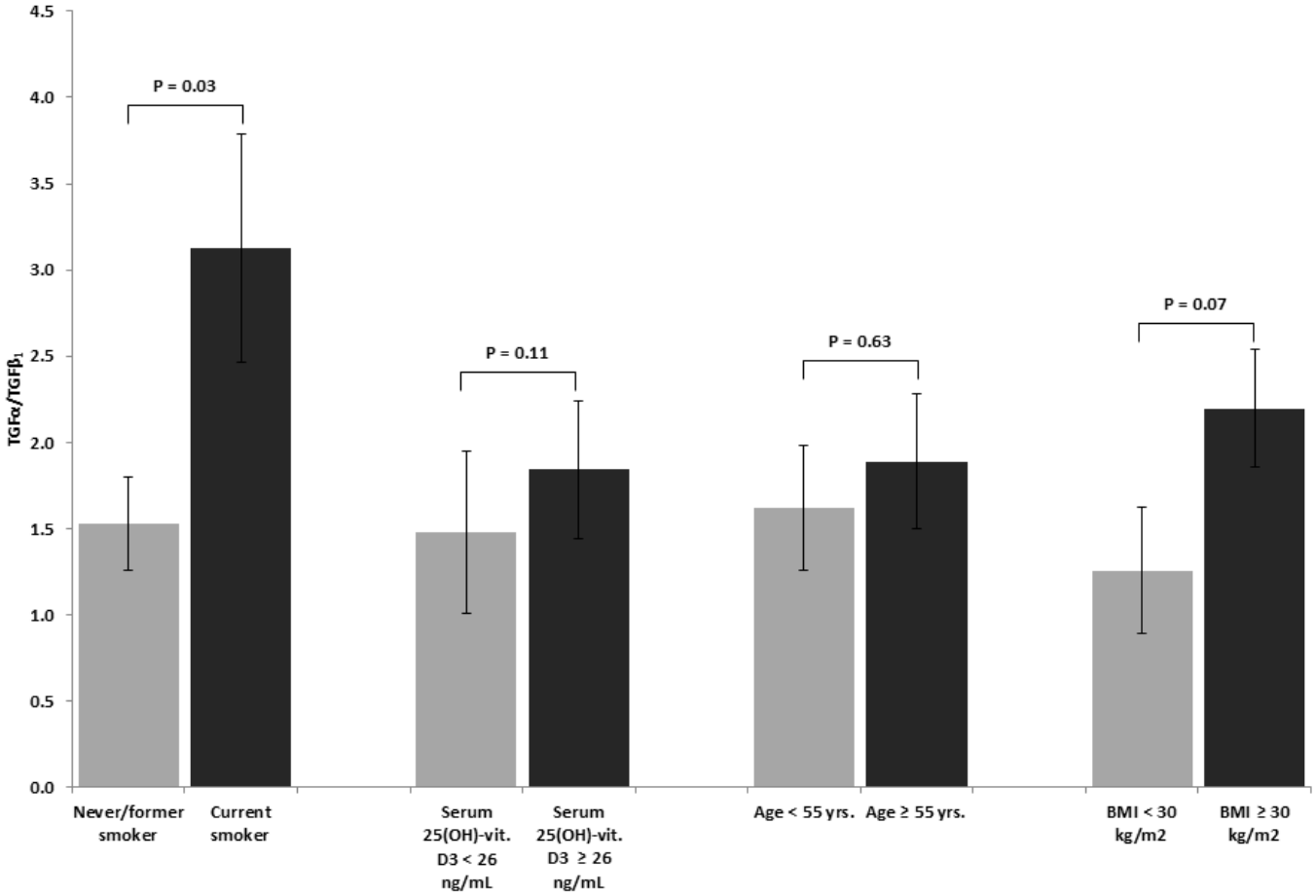

Figure 5. 3. TGFα/TGFβ_1 expression ratio in biopsies of normal-looking rectal mucosa according to selected risk factors for colorectal neoplasms. **Abbreviations**: TGFα, transforming growth factor alpha; TGFβ_1, transforming growth factor beta 1; BMI, body mass index

Supplementary Table 5.1. Selected characteristics of incident, sporadic colorectal adenoma cases and healthy controls, all MAP II participants

Characteristics [a]	N (cases/controls)	Cases (n = 48)	Controls (n = 147)	P-value [b]
Demographics, medical history, habits, anthropometrics				
Age (yrs.)	48/147	56.6 ± 6.7	55.5 ± 7.7	0.39
Male (%)	48/147	56.3	49.7	0.51
Caucasian (%)	47/146	97.9	94.5	0.69
1º relative with colorectal cancer (%)	45/136	15.6	21.3	0.52
Take NSAID ≥1 week (%)	47/146	31.9	37.0	0.60
Take Aspirin ≥1 week (%)	47/146	40.4	40.4	1.00
Use HRT (females) (%)	19/71	89.5	67.6	0.08
Current smoker (%)	48/147	22.9	14.3	0.18
Consume alcohol currently (%)	48/147	66.7	59.9	0.49
Body mass index (kg/m^2)	47/146	30.7 ± 7.2	29.7 ± 7.0	0.43
Waist-to-hip ratio	47/145	0.94 ± 0.09	0.91 ± 0.12	0.14
Moderate/vigorous physical activity (METs/day)	48/147	25.1 ± 18.9	24.7 ± 18.3	0.90
Serum 25(OH)D_3, ng/mL	40/134	28.0 ± 12.7	27.5 ± 11.2	0.84
Dietary intakes [c]				
Total energy (kcal/day)	48/147	1936 ± 744	1605 ± 541	< 0.01
Total fat (g/day)	48/147	66.3 ± 16.0	64.9 ± 15.4	0.72
Saturated fat (g/day)	48/147	21.7 ± 6.0	21.1 ± 5.2	0.47
Total folate (µg/day) [d]	48/147	496.5 ± 233.9	524.4 ± 272.8	0.53
Total fiber (g/day) [d]	48/147	15.4 ± 5.5	15.2 ± 5.0	0.78
Total calcium intake (mg/day) [d]	48/147	916.6 ± 472.6	904.6 ± 466.3	0.88
Total vitamin D intake (IU/day) [d]	48/147	342.5 ± 280.2	347.5 ± 293.8	0.92

Fruits and vegetables (servings/wk.)	48/147	29.8 ± 15.6	26.0 ± 12.1	0.12
Red meat (servings/wk.)	48/147	5.9 ± 3.9	5.2 ± 4.4	0.35
Processed meat (servings/wk.)	48/147	2.7 ± 2.5	2.3 ± 2.2	0.31

Chapter 6. Effects of calcium and vitamin D_3 on transforming growth factors in rectal mucosa of sporadic colorectal adenoma patients: a randomized controlled trial

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Abstract

Transforming growth factor alpha (TGFα) and TGFβ_1 are growth-promoting and -inhibiting autocrine/paracrine growth factors, respectively, that may 1) affect risk for colorectal cancer and 2) be modifiable by anti-proliferative exposures. The effects of supplemental calcium and vitamin D_3 on these two markers in the normal-appearing colorectal mucosa in humans are unknown. We conducted a pilot, randomized, double-blind, placebo-controlled, 2x2 factorial clinical trial (n=92; 23/treatment group) of calcium 2 g and/or vitamin D_3 800 IU/day versus placebo over 6 months. TGFα and TGFβ_1 expression was measured in biopsies of normal-appearing rectal mucosa using automated immunohistochemistry and quantitative image analysis at baseline and 6-month follow-up. In the calcium, vitamin D_3, and calcium plus vitamin D_3 groups relative to the placebo group 1) the mean overall expression of TGFβ_1 increased by 14% (P=0.25), 19% (P=0.17), and 22% (P=0.09); 2) the ratio of TGFα expression in the upper 40% (differentiation zone) to that in the lower 60% (proliferation zone) of the crypts decreased by 34% (P=0.11), 31% (P=0.22), and 26% (P=0.33); and 3) the TGFα/TGFβ_1 ratio in the upper 40% of the crypts decreased by 28% (P=0.09), 14% (P=0.41), and 22% (P=0.24), respectively. These preliminary results, although not statistically significant, suggest that supplemental calcium and vitamin D_3 may increase TGFβ_1 expression and shift TGFα expression downward from the differentiation to the proliferation zone in the crypts in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients, and support further investigation in a larger clinical trial.

Introduction

Colorectal cancer remains the second most common cause of cancer death in the United States (362), and it is widely accepted that most colorectal carcinomas develop from adenomas (6). Modifications in diet and lifestyle have been proposed to reduce colorectal cancer incidence and mortality (560). However, clinical trials of diet and lifestyle interventions with colorectal cancer incidence or mortality as the endpoints are limited due to the extended time to develop colorectal cancer and the large sample sizes and high costs involved. Therefore, modifiable pre-neoplastic biomarkers of risk for colorectal neoplasms that could be used as surrogate endpoints to investigate the potential efficacy of preventive interventions in short-term clinical trials are needed.

Calcium and vitamin D are two plausible and evidentially well-supported dietary preventive agents against colorectal neoplasms. Observational epidemiological studies have consistently found calcium intake to be inversely associated with colorectal cancer risk (450), and calcium supplementation reduced sporadic colorectal adenoma recurrence (561). Higher serum 25-OH-vitamin D concentrations, in a limited number of observational studies, have been consistently associated with lower risk for colorectal cancer (562) and adenomas (563). The three most prominent mechanisms of calcium against colorectal cancer include protection of the colorectal mucosa against bile acids, direct effects on the cell cycle, and modulation of E-cadherin and β-catenin expression in the APC colon carcinogenesis pathway (456). The four most prominent mechanisms for vitamin D include bile-acid catabolism, direct effects on the cell cycle, growth-factor signaling, and immunomodulation (456). As with calcium, these potential mechanisms are probably complementary. Indeed, we previously reported that, in the same clinical trial reported herein, calcium and/or vitamin D_3 supplementation favorably modulated biomarkers of their metabolism (559), apoptosis (557), proliferation and differentiation

(553), DNA damage (554), DNA mismatch repair (555), inflammation (558), and APC-β-catenin signaling (556).

Transforming growth factor alpha (TGFα) and transforming growth factor beta 1 ($TGF\beta_1$) are autocrine/paracrine growth factors that are classically thought of as potent promoters and inhibitors of cell growth, respectively, in normal tissues (476, 477), and likely contribute to or at least affect colorectal carcinogenesis (478). To our knowledge, no other human studies have assessed the effects of calcium and vitamin D_3 supplementation on the expression of these two markers in the normal human colorectal mucosa. The goal of the present study was to estimate the effects of supplemental calcium and vitamin D_3 on TGFα and $TGF\beta_1$ expression in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients.

Materials and Methods

This study was approved by the Emory University Institutional Review Board. Written informed consent was obtained from each study participant.

Participant population

The detailed protocol of this pilot, randomized, double-blind, placebo-controlled, 2x2 factorial clinical trial was published previously (557). Briefly, all participants were recruited from patients attending the Digestive Diseases Clinic of The Emory Clinic, Emory University. Inclusion criteria included 30 to 75 years of age, in general good health, capable of informed consent, and a history of at least one pathology-confirmed adenomatous colorectal polyp within the past 36 months. Exclusion criteria included contraindications to calcium or vitamin D supplementation or rectal biopsy procedures,

and medical conditions, habits, or medication usage that potentially could interfere with interpretation of the study results (557).

Clinical trial protocol

The detailed protocol for the clinical trial was published previously (557). Briefly, between April 2005 and January 2006, 105 potential participants attended an eligibility visit during which they were interviewed; signed a consent form; their medication and nutritional supplement bottles reviewed; questionnaires (on sociodemographics, medical history, medication and nutrition supplement use, lifestyle, family history, and others) completed; and a blood sample procured. Diet was assessed with a semi-quantitative food frequency questionnaire (530). Medical and pathology records were reviewed. Those still eligible and willing to participate entered a 30-day placebo run-in trial. Participants (n = 92) with no significant perceived side effects and who took ≥ 80% of their assigned tablets were randomly assigned, stratified by sex and NSAID use, to the following four treatment groups (n = 23/group): placebo, 2.0 g elemental calcium supplementation (as calcium carbonate in equal doses twice daily), 800 IU vitamin D_3 supplementation (400 IU twice daily), and 2.0 g elemental calcium plus 800 IU vitamin D_3 supplementation. Study tablets were custom manufactured by Tishcon Corp. (Salisbury, MD). The corresponding supplement and placebo pills, which were taken with meals, were identical in size, appearance, and taste. The chosen calcium dose was at the upper range at which no side effects would be likely, and the chosen vitamin D dose was twice the Recommended Daily Allowance (RDA) for most adults at the time the study was conceived (2002). Additional details on the rationale for the doses and forms of calcium and vitamin D_3 supplements were previously published (557).

Participants were instructed to maintain their usual diet and not take any nutritional supplements that they were not taking at the time of entry into the study. Over the 6-month treatment period, participants attended two follow-up visits, which were 1 and 6 months after randomization. At both follow-up visits, participants were asked about adherence and adverse events by questionnaire, interview, and pill count. At the final 6-month follow-up, participants again underwent a rectal biopsy and provided a blood sample.

Six approximately 1.0 mm-thick biopsy specimens were taken from the normal appearing rectal mucosa 10 cm above the level of the external anal aperture through a rigid sigmoidoscope with a jumbo cup flexible biopsy forceps. No biopsy was taken within 4.0 cm of a polypoid lesion. Biopsies were placed onto a strip of bibulous paper and immediately placed in phosphate buffered saline, oriented, transferred to 10% normal buffered formalin for 24 hours, and then transferred to 70% ethanol. Then, biopsies were processed and embedded in paraffin blocks within a week (2 blocks of 3 biopsies each per participant, per biopsy visit), cut and stained within another 4 weeks, and analyzed within another 4 weeks.

Immunohistochemistry protocol

From each block, five slides, with 4 levels of 3.0 μm-thick biopsy sections (taken 40 μm apart) on each slide, were prepared for each antigen, yielding a total of 20 levels for each antigen. Heat-mediated antigen retrieval was performed by steaming the slides in a preheated Pretreatment Module (Lab Vision Corp.) with 100x Citrate Buffer (pH 6.0; DAKO S1699, DAKO Corp.) for 40 mins. Then, the slides were immunohistochemically processed in a DAKO Automated Immunostainer (DAKO Corp.) using a labeled streptavidin-biotin method (TGFα antibody manufactured by Calbiochem, catalog No.

GF10, dilution 1:100; TGFβ_1 antibody manufactured by Santa Cruz, catalog No. sc-146, dilution 1:75), but not counterstained. The processed slides were coverslipped with a Leica CV5000 Coverslipper (Leica Microsystems, Inc.). Each staining batch contained approximately equal numbers of participants from each treatment group. Positive and negative control slides were included in each staining batch.

Protocol for quantifying labeling optical densities of immunohistochemically detected biomarkers in normal rectum crypts ("scoring")

A quantitative image analysis method to quantify the labeling optical densities ("expression") of the immunohistochemically-detected biomarkers in normal rectal crypts was described in detail previously (555, 557). Briefly, the image analysis unit was a "hemicrypt", defined as one side of a rectal crypt bisected from base to rectal lumen surface. A "scorable" hemicrypt was defined as an intact hemicrypt extending from the muscularis mucosa to the colon lumen. Before image analysis, staining adequacy was checked by examining the batch's negative and positive control slides.

Before scoring, the scorer, who was blinded to the intervention assignment, selected the two of the three biopsies with the greatest number of scorable hemicrypts, captured background correction images for each slide, and captured 16-bit grayscale images of crypts at 200x magnification. Then, the scorer traced the borders of the "hemicrypt" in the image analysis program (**Figure 6.1**). The program then segmented the traced hemicrypt, and the background-adjusted optical density of the labeling across the whole hemicrypt and within each segment was measured and exported to a Microsoft Access database. The goal was to score 16 to 20 hemicrypts per biopsy visit for each biomarker.

Reliability was assessed by selecting samples of previously analyzed slides (10%) to be re-analyzed by the same scorer. The scorer was blinded to the selection. Intra-reader reliability for TGFα and TGFβ_1 was above 0.90 throughout.

Statistical analysis

All statistical analyses were performed using SAS 9.3 statistical software (SAS Institute Inc.). A P value ≤ 0.05 (two-sided) was considered statistically significant. Treatment groups were assessed for comparability of characteristics at baseline by the Fisher's exact test for categorical variables and analysis of variance for continuous variables. Slide scoring reliability was analyzed using intra-class correlation coefficients.

The mean biomarker expression in each study participant, at baseline and 6-month follow-up, was calculated by averaging the biomarker expression on all the analyzed hemicrypts. To adjust for possible staining batch effects, batch-standardized mean biomarker expression was calculated by dividing an individual participant's biomarker expression by the mean biomarker expression on all the participants in the same batch (557). To represent distinct functional zones of rectal crypts, the upper 40% of the crypts (differentiation zone) and the lower 60% of the crypts (proliferation zone) were selected *a priori* as measures of crypt biomarker distribution (564). A TGFα/TGFβ_1 ratio was calculated as an indicator of the balance between the growth-promoting and -inhibiting factors by dividing the mean batch standardized level of TGFα by that of TGFβ_1; a higher ratio, thus, would indicate a more pro-growth balance.

The distributions of batch-standardized TGFα and TGFβ_1 labeling optical densities along the full length of the hemicrypts were graphically plotted and modeled using the LOESS procedure. First, each hemicrypt was standardized to 50 sections. Then, the batch-standardized average of each section across all hemicrypts was

calculated and predicted by the LOESS model separately for each treatment group, by visit. The results were graphically plotted along with smoothing lines. Although the plots illustrate the distribution of expression, they do not provide a complete analysis of treatment effects because they do not account for changes in the placebo group. Based on graphical assessments, an upper 40% to lower 60% ratio was created *a posteriori* for TGFα.

Primary analyses were based on randomization treatment assignment, regardless of adherence status (intent-to-treat analysis). Treatment effects were evaluated by assessing the differences in the batch-adjusted biomarker expression from baseline to the 6-month follow-up between participants in the active treatment groups and those in the placebo group by a repeated-measures linear MIXED effects model. The model included the intercept, treatment groups, visit (baseline and follow-up), and a treatment group by visit effect interaction term. Because optical density is measured in arbitrary units, to provide perspective on the magnitude of the treatment effects, we also calculated relative effects. The relative effect was calculated as the (treatment group at follow-up / treatment group at baseline) / (placebo group at follow-up / placebo group at baseline). The relative effect provides a conservative estimate of the proportional change in the treatment group relative to that in the placebo group, and its interpretation is somewhat analogous to that of an odds ratio (e.g., a relative effect of two would mean that the proportional change in the treatment group was two times that in the placebo group) (557, 565).

Results

Characteristics of study participants

The mean age of study participants was 61 years, 70% were men, 71% were white, and 20% had a family history of colorectal cancer in a first degree relative. The treatment groups were balanced on baseline characteristics except that there were higher proportions of regular aspirin use in the calcium and calcium plus vitamin D groups (**Table 6.1**). Average adherence to visit attendance was 92% and did not significantly differ among the four treatment groups. On average, at least 80% of pills were taken by 93% of participants at the first follow-up visit and by 84% of participants at the final follow-up visit. No adverse events were attributed to study procedures or treatments. Seven participants (8%) were lost to follow-up. Dropouts included one person from the vitamin D_3 supplementation group and two from each of the other three groups. Adequate biopsy specimens for image analysis for TGFα and TGFβ_1 were available for 84 and 86 participants at baseline and for 84 and 83 participants after a 6-month follow-up, respectively.

Baseline serum 25-OH-vitamin D concentrations did not differ between the four treatment groups. At the 6-month follow-up, serum 25-OH-vitamin D concentrations had increased 60% ($p<0.0001$) and 56% ($p<0.0001$) in the vitamin D_3 and calcium plus vitamin D_3 groups, respectively, relative to placebo (557); however, mean post-treatment serum 25-OH-vitamin D concentrations remained below 30 ng/ml in all treatment groups (17.9, 23.2, 29.5, and 28.5 ng/ml in the placebo, calcium, vitamin D, and calcium plus vitamin D groups, respectively).

Effects on TGFα

At baseline, TGFα expression in the rectal mucosa did not differ significantly among the four treatment groups (**Table 6.2**). In the graphical assessment (**Figure 6.2**), TGFα expression in all active treatment groups during the trial appeared to decrease in

the approximate upper 40% (differentiation zone) of the crypts, but increase in the approximate lower 60% (proliferative zone) of the crypts. In the numerical assessment (Table 2), while TGFα expression in the whole crypts did not change substantially in the calcium, vitamin D, and calcium plus vitamin D groups, it decreased by 14% (P = 0.35), 2% (P = 0.86), and 1% (P = 0.93), respectively, in the upper 40% of the crypts, but increased by 29% (P = 0.30), 38% (P = 0.17), and 25% (P = 0.32) in the bottom 60% of the crypts. To quantify the apparent TGFα expression crypt zone shift seen in the graphical assessment, we created an upper 40% to lower 60% ratio and found that it decreased by 34% (P = 0.11), 31% (P = 0.22), and 26% (P = 0.33) in the calcium, vitamin D, and calcium plus vitamin D groups, respectively, relative to the placebo group.

Effects on TGFβ_1

At baseline, TGFβ_1 expression in the rectal mucosa did not differ significantly among the four treatment groups (Table 6.2). In the graphical assessment (**Figure 6.3**), TGFβ_1 expression in all active treatment groups during the trial appeared to increase relatively uniformly throughout the lengths of the crypts. In the numerical assessment (Table 2), the mean overall expression of TGFβ_1 in the whole crypts increased by 14% (P = 0.25), 19% (P = 0.17), and 22% (P = 0.09) in the calcium, vitamin D, and calcium plus vitamin D groups, respectively, relative to the placebo group. Reflecting the graphical findings, the respective changes in the differentiation and proliferation zones of the crypts were similar to those for the whole crypts (Table 2).

Effects on TGFα/TGFβ_1 ratios

After 6 months of treatment, the TGFα/TGFβ_1 ratio in the whole crypts decreased by 14% (P = 0.46) and 11% (P = 0.09) in the calcium and calcium plus vitamin D groups, respectively, but increased by 2% (P = 0.93) in the vitamin D group, relative to the

placebo group. In the upper 40% of the crypts, the TGFα/TGFβ_1 ratio decreased by 28% (P = 0.09), 14% (P = 0.41), and 22% (P = 0.24) in the calcium, vitamin D, and calcium plus vitamin D groups, respectively, relative to the placebo group; however, in the bottom 60% of the crypts, the TGFα/TGFβ_1 ratio increased by 16% (P = 0.61) and 30% (P = 0.36) in the calcium and vitamin D groups, but decreased by 2% (P = 0.94) in the calcium plus vitamin D group, relative to the placebo group (Table 6.2). A data-derived TGFα upper 40% to lower 60%/TGFβ_1 ratio that was created *a posteriori* as a best discriminator of the intervention effects decreased by 48% (P = 0.08), 44% (P = 0.12), and 35% (P = 0.28) in the calcium, vitamin D, and calcium plus vitamin D groups, respectively, relative to the placebo group.

Sensitivity analyses

Neither multiple imputation to impute missing observations nor adjusting the analyses for baseline aspirin and/or NSAID use, serum 25-OH-vitamin D concentrations, and calcium intake appreciably changed our findings.

Discussion

Our preliminary results, although not statistically significant, suggest that calcium and/or vitamin D_3 supplementation over six months may increase the overall expression of TGFβ_1 and shift TGFα expression downward into the proliferation zone in the colorectal crypts in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients, and support a larger study to investigate this hypothesis further as well as other investigations of whether calcium and vitamin D_3 may reduce colorectal cancer risk, in part, by modulating growth factors.

Currently, there are no widely accepted pre-neoplastic biomarkers of risk for colorectal neoplasms. One potential biomarker is colorectal epithelial cell proliferation, which is regulated by growth factors (476). Compared to patients at lower risk for colon cancer, patients with colon cancer and patients in every category known to be at higher risk for colon cancer on average, exhibit in their normal-appearing mucosa both an increased colorectal epithelial cell proliferation rate and an extension of the colon crypt proliferative zone from the lower (basal) 60% of the crypt to include the upper (luminal) 40% of the crypt (525). In patients with previous colon cancer or sporadic adenomas, these changes also predicted adenoma recurrence (545, 566).

It was previously reported that, in the normal colorectal mucosa, immunohistochemically-detected TGFα was denser in the upper one-third to two thirds of colonic crypts (479-481) (differentiation zone), and our staining pattern was consistent with these findings. Consistent with its pro-growth and hyperproliferative role, TGFα expression was found to be greater in the normal-appearing rectal mucosa of sporadic colorectal adenoma patients than in adenoma-free patients (526), in colorectal adenomas and cancer (532, 533), and in the blood of colorectal cancer patients (534,

535). In our study we found no substantial changes in the overall expression of TGFα in whole crypts in the normal-appearing colorectal mucosa after calcium and/or vitamin D_3 supplementation; however, our results suggest that calcium and/or vitamin D_3 supplementation may shift TGFα expression downward into the colorectal crypt proliferation zone. These findings are consistent with those we previously reported on the effects of calcium and vitamin D on cell proliferation markers. In the first trial (n = 192), we found that calcium supplementation, without changing the proliferation rate, substantially, statistically significantly decreased the proportion of proliferating cells in the upper 40% of the crypts relative to the whole crypts in the rectal mucosa of sporadic adenoma patients (565). From the same trial reported herein, we found that calcium plus vitamin D_3 may shift downwards the expression of hTERT, a marker of long-term proliferation, in the crypt proliferation zone without affecting its overall expression (553). Furthermore, reports from other groups also indicated that calcium could decrease cell proliferation in the upper 40% of the crypt, but not the overall cell proliferation rate (567, 568). The findings from the present study suggest that calcium and vitamin D may promote confinement of proliferating cells to the proliferation zone, at least in part, via modulating TGFα expression.

While the mechanism for the shift of TGFα expression downwards into the proliferation zone by calcium and vitamin D is unclear, this downward shift may reduce risk for colorectal neoplasms. First, a downward shift of TGFα may reduce the number of dividing cells in the luminal pole of the crypt. DNA is more susceptible to damage during cell division, and cells at the luminal pole of the crypt are more likely to be exposed to carcinogens in the colon lumen. Second, it has been proposed that colorectal adenomas originate from the upper crypt surface (569), and TGFα was found to be the main survival factor for early adenoma cells against apoptosis (570), so a

downward shift of TGFα may decrease the likelihood of developing colorectal adenomas. Alternatively, it is possible that the downward shift in TGFα could stimulate initiated cells in the proliferation zone to grow faster and thus promote colorectal carcinogenesis; however, given that supplemental calcium statistically significantly reduced adenoma recurrence in a large, randomized controlled trial (451), this seems less likely.

No previous human studies tested the effects of supplemental calcium and/or vitamin D on TGFα expression in the normal colorectal mucosa; however, in two small (n = 13 and 10) clinical trials, cellulose (480) and 81 mg of aspirin (571) statistically significantly decreased the percentage of TGFα expression-positive cells in the normal colorectal mucosa of adenoma patients.

$TGF\beta_1$ immunoreactivity was previously reported to localize mainly in the upper third of the crypts of the normal colorectal mucosa (483). $TGF\beta_1$ signaling, which is complex in cancer progression, regulates cell proliferation, apoptosis, autophagy, inflammation, tumor angiogenesis, and metastasis (482). A dual role of $TGF\beta_1$ has been proposed, because $TGF\beta_1$ suppresses the growth of normal epithelial cells but promotes tumor metastasis in later stages of cancer (482). In a mouse model, the absence of $TGF\beta_1$ expression promoted the progression from hyperplasia to adenoma and allowed the development of carcinoma (486). Our study suggests that calcium and/or vitamin D_3 supplementation may increase $TGF\beta_1$ expression in the normal-appearing colorectal mucosa where $TGF\beta_1$ should function as a cell growth suppressor. Findings from studies in cell lines and animals suggested that calcium (572) and vitamin D (573-576) could induce $TGF\beta_1$ expression, and our results are consistent with these findings.

No previous human studies tested the effect of supplemental calcium and/or vitamin D on $TGF\beta_1$ expression in the normal colorectal mucosa, but one small (n = 39) randomized, controlled trial in multiple sclerosis patients found that 1,000 IU of supplemental vitamin D daily over six months statistically significantly increased serum $TGF\beta_1$ levels (577). The molecular mechanism by which vitamin D induces $TGF\beta_1$ expression is unclear. While one study identified two vitamin D response elements (VDREs) in the *TGFβ₂* gene promoter region (578), it is unclear whether the *TGFβ₁* gene promoter region contains a VDRE. Also, vitamin D stimulates activator protein-1 (AP-1) expression and enhances its binding to DNA (579), and AP-1 is a mediator of $TGF\beta_1$ autoinduction by binding to specific promoter elements in the *TGFβ₁* gene (580).

Our study had several limitations. First, it was a pilot study with limited statistical power, especially for stratified analyses. Second, we collected biopsies only from the rectum; however, previous studies found that levels of cell proliferation markers in the rectum reflected those in other areas of the colon (581, 582). Third, we measured protein expression rather than protein activity, although measuring either is likely to represent protein function in normal tissue. Fourth, the dose of vitamin D supplementation used in the present study may have been insufficient; however, when the study was designed in 2002, 800 IU/day at twice the RDA was considered a bold choice. Finally, our participants were limited to sporadic colorectal adenoma patients and caution should be taken when generalizing our results to other populations.

The strengths of our study are: (i) to our knowledge, it is the first randomized, double-blind, placebo-controlled clinical trial to test the effects of calcium and/or vitamin D_3 supplementation on TGFα and $TGF\beta_1$ expression in the normal colorectal epithelium in sporadic adenoma patients; (ii) the high protocol adherence; and (iii) the automated immunostaining and newly designed image analysis software that allowed quantification

of TGFα and TGFβ_1 expression overall as well as their distributions within the colorectal crypts.

In summary, the results of this pilot clinical trial, although not statistically significant, suggest that calcium and/or vitamin D_3 supplementation over 6 months may increase overall TGFβ_1 expression, and shift downwards ("normalize") the expression of TGFα from the differentiation zone to the proliferation zone in the normal colorectal mucosa of sporadic colorectal adenoma patients, and support further investigation in a larger clinical trial. Taken together with previous literature that suggests that anti-carcinogenic effects of supplemental calcium and vitamin D_3 may, in part, depend on the ability of these agents to favorably modulate the expression of the TGFα and TGFβ_1 expression, our results also support further investigation of 1) calcium and vitamin D_3 as chemopreventive agents against colorectal neoplasms and 2) whether TGFα and TGFβ_1 expression could be used as modifiable biomarkers and surrogate endpoints to investigate the potential efficacy of preventive interventions against colorectal neoplasms.

The authors' contributions were as follows: R.M.B. designed and developed the research and conducted the study; R.M.B, W.D.F., and Q.L. developed the methodology; R.M.B. oversaw the study and provided administrative, technical, or material support; C.R.D., A.G.G.F., and R.E.R. collected the data; H.T., T.U.A., and R.M.B. performed data analyses; H.T., R.M.B., W.D.F., and T.U.A. drafted the manuscript; and all authors reviewed and approved the final content of the manuscript.

Figure legends

Figure 6.1. Process of quantitative image analysis. **A**, identifying scorable crypts; **B**, tracing the hemicrypt; **C**, automated sectioning of the trace; and **D**, automated quantification of TGFα labeling optical density in the whole hemicrypt and each section.

Figure 6.2. Standardized transforming growth factor alpha (TGFα) expression (labeling optical density) along normal colorectal crypts by treatment group at baseline and 6-month follow-up. The distributions were modeled and graphically plotted using the LOESS procedure.

Figure 6.3. Standardized transforming growth factor beta 1 ($TGF\beta_1$) expression (labeling optical density) along normal colorectal crypts by treatment group at baseline and 6-month follow-up. The distributions were modeled and graphically plotted using the LOESS procedure.

Table 6.1. Selected baseline characteristics of the study participants [a] (n = 92)

Characteristics	Treatment Group: Placebo (n = 23)	Calcium (n = 23)	Vitamin D_3 (n = 23)	Calcium + Vit. D_3 (n = 23)	*P*-value [b]
Demographics, medical history, habits, anthropometrics					
Age, years	58.5 (8.2)	61.9 (8.2)	60.2 (8.1)	62.1 (7.5)	0.39
Men (%)	70	70	70	70	1.00
White (%)	74	83	65	61	0.40
College graduate (%)	65	64	57	44	0.53
History of colorectal cancer in 1° relative (%)	17	30	17	13	0.60
Take NSAID [c] regularly [d] (%)	22	13	4	13	0.43
Take aspirin regularly (%)	22	52	30	57	0.05
If woman (n = 28), taking estrogens (%)	4 (14)	4 (14)	4 (14)	9 (29)	1.00
Current smoker (%)	9	4	0	0	0.61
Take multivitamin (%)	30	30	26	39	0.86
Body mass index (BMI), kg/m^2	30.6 (7.2)	29.4 (5.5)	28.9 (5.56)	31.6 (6.0)	0.44
Mean dietary intakes [e]					
Total energy intake, kcal/d	1596 (528)	1788 (691)	1848 (821)	1845 (752)	0.59
Total [f] calcium, mg/d	619 (308)	746 (335)	843 (526)	824 (714)	0.41
Total [f] vitamin D, IU/d	277 (230)	336 (202)	360 (317)	415 (315)	0.40
Total fat, gm/d	67 (32)	72 (35)	70 (32)	74 (28)	0.89
Dietary fiber, gm/d	15 (7)	17 (9)	18 (9)	17 (11)	0.70
Alcohol intake, gm/d	9 (14)	11 (15)	14 (18)	10 (20)	0.76
Total serum vitamin D					
25-OH-vitamin D, ng/mL	20.4 (7.6)	25.7 (7.6)	21.0 (8.3)	20.9 (9.6)	0.12

[a] Data are given as means (SD) unless otherwise specified.

[b] By Fisher's exact test for categorical variables, and ANOVA for continuous variables.

[c] Nonsteroidal anti-inflammatory drug.

[d] At least once a week.

[e] All nutrients energy adjusted using residual method

[f] Diet plus supplements.

Table 6.2. Standardized expression of transforming growth factor alpha (TGFα) and transforming growth factor beta 1 ($TGF\beta_1$) in the normal-appearing colorectal mucosa during the clinical trial

Treatment Group	Baseline n	Baseline Mean	Baseline Std Err	Baseline P	6-mo follow-up n	6-mo follow-up Mean	6-mo follow-up Std Err	6-mo follow-up P	Absolute Rx effect n	Absolute Rx effect Rx effect [a]	Absolute Rx effect Std Err	Absolute Rx effect P [b]	Relative Effect [c]
A. TGFα													
Whole crypts													
Placebo	20	1.06	0.13		21	1.00	0.04		18	0	.	.	1.00
Calcium	23	1.00	0.08	0.71	21	0.97	0.08	0.74	21	0.03	0.16	0.87	1.03
Vitamin D_3	20	0.96	0.09	0.53	21	0.98	0.06	0.85	19	0.09	0.16	0.61	1.09
Ca + Vit. D_3	21	0.98	0.14	0.62	21	1.01	0.07	0.88	19	0.09	0.16	0.59	1.10
Upper 40% of crypts													
Placebo	20	0.59	0.07		21	0.53	0.02		18	0	.	.	1.00
Calcium	23	0.67	0.06	0.42	21	0.52	0.04	0.79	21	-0.09	0.10	0.35	0.86
Vitamin D_3	20	0.61	0.06	0.84	21	0.54	0.03	0.89	19	-0.01	0.10	0.86	0.98
Ca + Vit. D_3	21	0.59	0.08	0.95	21	0.53	0.03	0.97	19	-0.01	0.10	0.93	0.99
Lower 60% of crypts													
Placebo	20	0.47	0.08		21	0.47	0.02		18	0	.	.	1.00
Calcium	23	0.34	0.04	0.13	21	0.44	0.04	0.53	21	0.10	0.10	0.30	1.29
Vitamin D_3	20	0.35	0.06	0.18	21	0.48	0.04	0.82	19	0.13	0.10	0.17	1.38
Ca + Vit. D_3	21	0.39	0.07	0.34	21	0.48	0.04	0.83	19	0.10	0.10	0.32	1.25
Top 40% to bottom 60% of the crypts													
Placebo	20	1.69	0.28		21	1.17	0.06		18	0.00	.	.	1.00
Calcium	23	2.79	0.45	0.07	21	1.27	0.07	0.29	21	-0.99	0.63	0.11	0.66
Vitamin D_3	20	2.53	0.43	0.18	21	1.20	0.07	0.74	19	-0.80	0.64	0.22	0.69
Ca + Vit. D_3	20	2.28	0.51	0.34	20	1.17	0.08	0.99	19	-0.59	0.64	0.33	0.74
B. $TGF\beta_1$													
Whole crypts													
Placebo	21	1.04	0.06		21	1.00	0.02		19	0	.	.	1.00
Calcium	22	1.03	0.06	0.95	21	1.13	0.07	0.12	20	0.14	0.13	0.25	1.14
Vitamin D_3	22	0.93	0.07	0.23	20	1.07	0.06	0.42	19	0.17	0.13	0.17	1.19
Ca + Vit. D_3	21	1.00	0.05	0.64	21	1.18	0.07	0.04	19	0.22	0.13	0.09	1.22
Upper 40% of crypts													
Placebo	21	0.39	0.02		21	0.38	0.01		19	0	.	.	1.00
Calcium	22	0.40	0.02	0.83	21	0.43	0.03	0.11	20	0.04	0.05	0.35	1.11
Vitamin D_3	22	0.35	0.03	0.25	20	0.41	0.03	0.34	19	0.07	0.05	0.15	1.20
Ca + Vit. D_3	21	0.39	0.02	0.86	21	0.45	0.02	0.03	19	0.08	0.05	0.12	1.20

Lower 60% of crypts												
Placebo	21	0.65	0.04		21	0.62	0.02		19	0	.	.	1.00
Calcium	22	0.64	0.04	0.80	21	0.70	0.03	0.10	20	0.10	0.07	0.18	1.16
Vitamin D_3	22	0.58	0.04	0.18	20	0.67	0.04	0.37	19	0.11	0.07	0.12	1.20
Ca + Vit. D_3	21	0.59	0.03	0.23	21	0.70	0.04	0.11	19	0.14	0.07	0.06	1.25
C. TGFα/TGFβ$_1$												
Whole crypts												
Placebo	19	1.03	0.12		21	1.00	0.03		17	0.00	.	.	1.00
Calcium	22	1.06	0.10	0.90	21	0.88	0.06	0.13	20	-0.14	0.19	0.46	0.86
Vitamin D_3	20	0.99	0.08	0.80	19	0.97	0.07	0.73	17	0.02	0.20	0.93	1.02
Ca + Vit. D_3	21	1.05	0.18	0.94	21	0.90	0.06	0.21	19	-0.12	0.19	0.55	0.89
Upper 40% of crypts												
Placebo	19	1.48	0.16		21	1.41	0.06		17	0.00	.	.	1.00
Calcium	22	1.85	0.20	0.19	21	1.26	0.09	0.21	20	-0.52	0.29	0.09	0.72
Vitamin D_3	20	1.71	0.16	0.43	19	1.39	0.10	0.88	17	-0.24	0.30	0.41	0.86
Ca + Vit. D_3	21	1.62	0.23	0.61	21	1.21	0.08	0.08	19	-0.35	0.30	0.24	0.78
Lower 60% of crypts												
Placebo	19	0.77	0.12		21	0.76	0.04		17	0.00	.	.	1.00
Calcium	22	0.55	0.07	0.19	21	0.63	0.06	0.08	20	0.09	0.18	0.61	1.16
Vitamin D_3	20	0.56	0.08	0.22	19	0.72	0.06	0.61	17	0.17	0.18	0.36	1.30
Ca + Vit. D_3	21	0.71	0.17	0.75	21	0.69	0.05	0.36	19	-0.01	0.18	0.94	0.98
D. TGFα upper 40% to lower 60%/TGFβ$_1$												
Placebo	19	1.62	0.30		21	1.19	0.07		17	0.00	.	.	1.00
Calcium	22	3.10	0.53	0.07	21	1.18	0.07	0.95	20	-1.49	0.85	0.08	0.52
Vitamin D_3	20	3.11	0.65	0.07	19	1.27	0.10	0.62	17	-1.40	0.87	0.12	0.56
Ca + Vit. D_3	20	2.46	0.66	0.31	20	1.16	0.21	0.90	18	-0.85	0.86	0.28	0.65

[a] Rx effect = [(treatment group follow-up) − (treatment group baseline)] − [(placebo group follow-up) − (placebo group baseline)].

[b] P value for difference between each active treatment group and placebo group from repeated-measures MIXED model.

[c] Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo follow-up) / (placebo baseline)]; interpretation similar to that for an odds ratio (e.g., a relative effect of 1.7 indicates a 70% proportional increase in the treatment group relative to that in the placebo group).

Abbreviations: TGFα, transforming growth factor alpha; TGFβ$_1$, transforming growth factor beta 1; Std Err, standard error

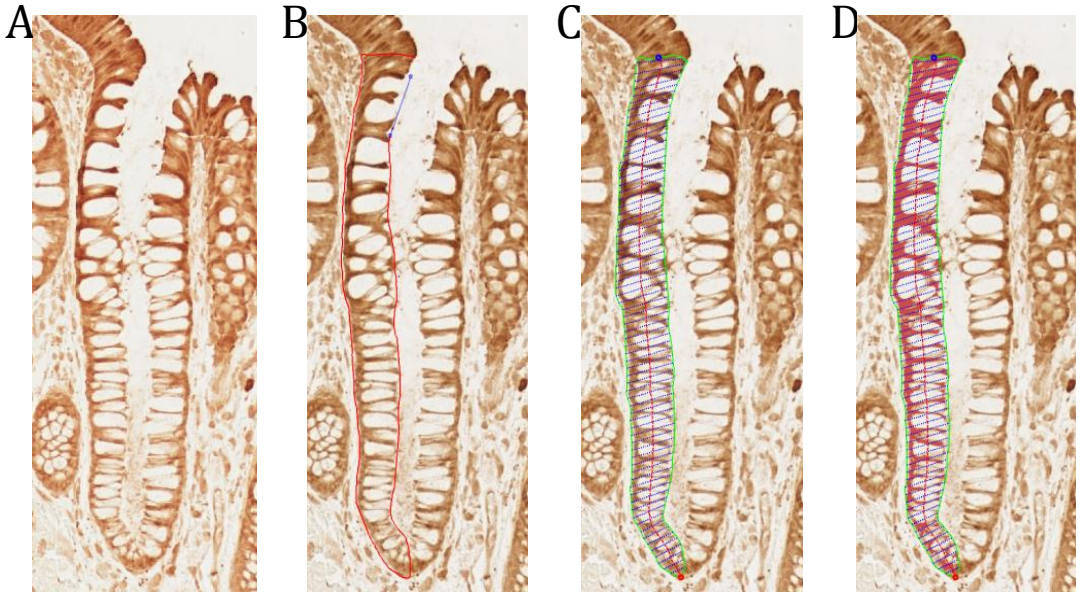

Figure 6.1. Process of quantitative image analysis. **A**, identifying scorable crypts; **B**, tracing the hemicrypt; **C**, automated sectioning of the trace; and **D**, automated quantification of TGFα labeling optical density in the whole hemicrypt and each section.

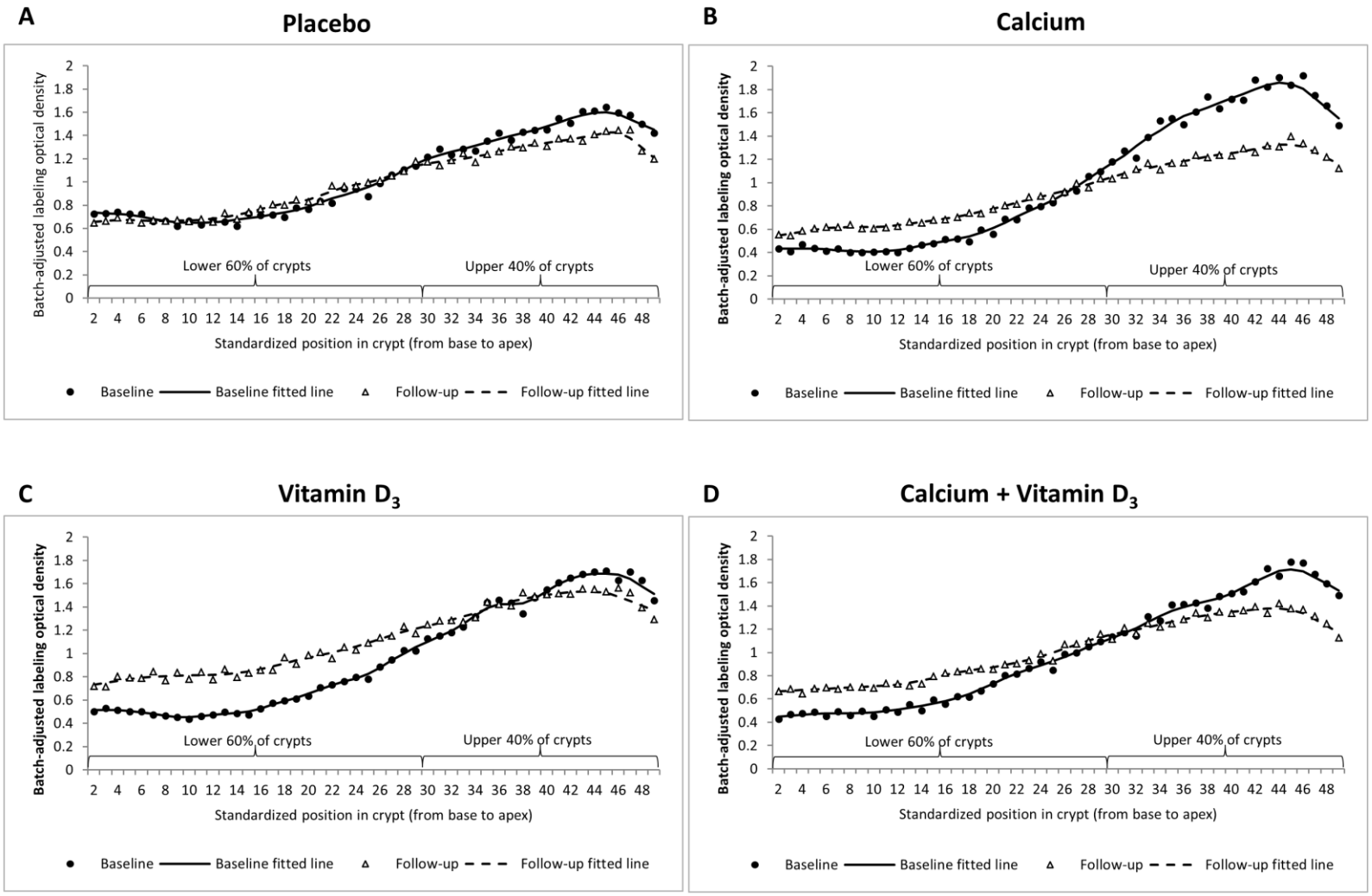

Figure 6.2. Standardized transforming growth factor alpha (TGFα) expression (labeling optical density) along normal colorectal crypts by treatment group at baseline and 6-month follow-up. The distributions were modeled and graphically plotted using the LOESS procedure.

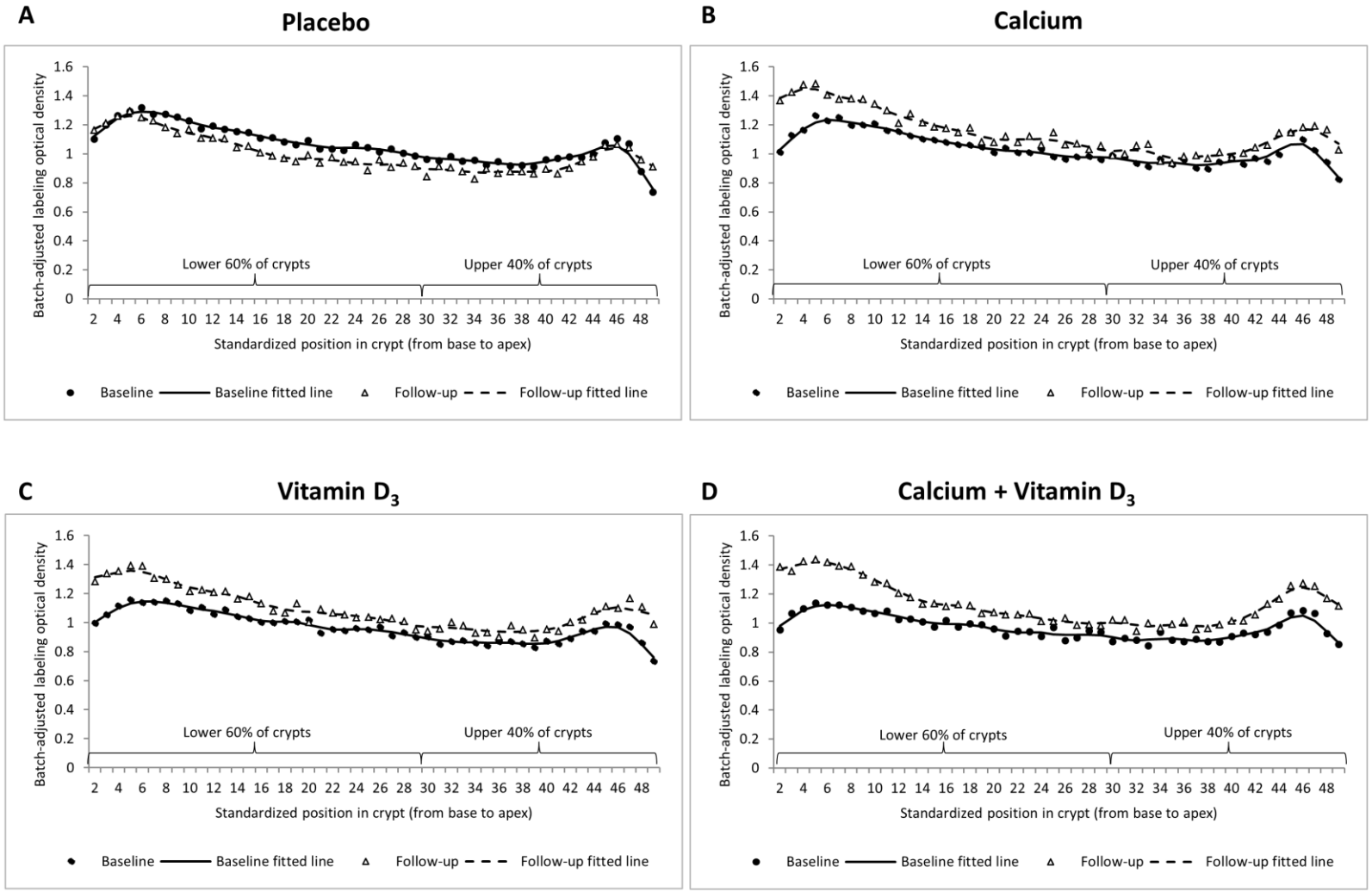

Figure 6.3. Standardized transforming growth factor beta 1 ($TGF\beta_1$) expression (labeling optical density) along normal colorectal crypts by treatment group at baseline and 6-month follow-up. The distributions were modeled and graphically plotted using the LOESS procedure.

Chapter 7. Conclusions and Implications

Two studies from a population-based, combined serologic/endoscopic screening program for gastric diseases, one study from a pilot colonoscopy-based case-control study, and one study from a pilot, randomized, double-blind, placebo-controlled, 2 x 2 factorial clinical trial were conducted to evaluate potential roles for plausible GC- and CRC-related biomarkers for 1) identifying precursor lesions, 2) risk stratification, and 3) surrogate endpoints in chemoprevention trials to assess the potential efficacy, safety, and optimal dose of interventions.

In the first dissertation study, a population-based, combined serologic/endoscopic screening program for gastric diseases, particularly GC, I found that serum PGII and anti-*H. pylori* IgG, especially combined, may provide adequate accuracy for aiding clinicians or large screening programs in identifying persons with abnormal gastric conditions that may require more invasive or intensive risk assessment or monitoring.

In the second dissertation study, a longitudinal study in which repeated gastroscopies with gastric mucosal biopsies and blood sample collections were conducted, I found that temporal changes in serum PGI, PGII, PGI/II ratio, and anti-*H. pylori* IgG levels (especially PGII and anti-*H. pylori* IgG combined) are associated with risk for progression of gastric precancerous lesions.

In the third dissertation study, a pilot colonoscopy-based case-control study, I found that the mean ratio of rectal TGFα to TGFβ_1 expression and mean rectal TGFα expression were higher in cases than in controls and associated with a higher risk of colorectal adenoma. In the fourth dissertation study, I used data from a pilot, randomized, double-blind, placebo-controlled, 2 x 2 factorial clinical trial of calcium 2,000

mg and/or vitamin D_3 800 IU daily over 6 months to investigate whether supplemental calcium and vitamin D could modulate the expression of TGFα and TGFβ_1 in the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients. I found that supplemental calcium and vitamin D increased TGFβ_1 expression and shifted TGFα expression downward into proliferation zone and decreased the mean ratio of rectal TGFα to TGFβ_1 expression.

The findings from my dissertation have important public and clinical implications. According to the most recent data, there were 0.95 million gastric cancer cases and 1.4 million colorectal cancer cases in 2012 (361), and new cases are expected to increase with expanding populations and increasing colorectal cancer risk in developing countries. The fact that most gastric cancer and colorectal cancer develop from precursors provides unique opportunities for gastric cancer and colorectal cancer prevention and early detection. It follows that effective management of precursors could lead to reduced gastric cancer and colorectal cancer incidence and mortality. The results of my dissertation suggest that plausible GC- and CRC-related biomarkers could play an important role in the management of gastric cancer and colorectal cancer precursors through their utility for identifying precursor lesions, risk stratification, and serving as surrogate endpoints in chemoprevention trials to assess the potential efficacy, safety, and optimal dose of interventions. Incorporation of hypothesis-based biomarkers into the management of gastric cancer and colorectal cancer precursors may help reduce the gastric cancer and colorectal cancer burden by promoting endoscopies (limited resources) in those at highest risk (i.e., most needed) and reducing endoscopies in those at lowest risk (i.e., least needed) and facilitating the process of identifying effective chemoprevention agents.

Chapter 8. Future Directions

In the first and second dissertation study, we investigated whether baseline serum biomarker profiles including PGI, PGII, PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG could be used to identify gastric cancer precursors and whether temporal changes in these biomarkers were associated with risk for gastric cancer precursors. To further clarify the role of including PGI, PGII, PGI/II ratio, gastrin-17, and anti-*H. pylori* IgG in the management of gastric cancer precursors, I propose to investigate whether baseline serum PGI, PGII, PGI/II ratio, anti-*H. pylori* IgG, and gastrin-17, individually and combined, are associated with developing gastric cancer among the 8,348 participants with precancerous lesions in the Zhuanghe Gastric Disease Screening Program. I also propose to investigate whether serum biomarker profiles combined with a baseline gastric histologic diagnosis are more strongly associated with risk for incident gastric cancer than are either alone.

Approximately 90% of all GCs originate from the glandular epithelium of the gastric mucosa (adenocarcinomas) (19, 20), making the gastric mucosa the most suitable place to begin the search for pre-neoplastic biomarkers of risk for GC. I propose to conduct an endoscopy-based, nested case-control study (n = 150 incident gastric cancer cases and 300 randomly selected matched controls) within the prospective Zhuanghe Gastric Disease Screening Program to investigate associations of selected hypothesis-based gastric tissue biomarkers with risk for incident, sporadic gastric adenocarcinoma. The candidate tissue biomarkers could be *H. pylori* constituents that mediate oncogenesis, human gastric epithelial cell proteins that interact with *H. pylori* constituents, markers of inflammation and oxidative stress/damage, DNA repair and affected markers, and markers of cell cycle (i.e., proliferation, differentiation, and apoptosis) and cell adhesion. These tissue markers would be measured using our

newly developed a new methodology, automated multiplex quantum dot immunohistochemistry with peptide controls and quantitative image analysis (mQD-IHC/qIA, or QIQI),(583) that allows detection and analysis of the amounts, distributions within the gastric epithelium architecture, and relationships to one another of 5-fold more hypothesis-based tissue biomarkers from a given amount of tissue.

Colorectal adenoma patients after adenoma removal still have a higher risk of developing adenomas and colorectal cancer than do those without adenomas, suggesting that normal-appearing tissue may retain components of risk. In the third dissertation study, we found that the mean ratio of rectal TGFα to TGFβ_1 expression and mean rectal TGFα expression were higher in adenoma cases than in controls, suggesting that they might be associated with the development of recurrent adenoma and CRC. I propose to validate whether the balance of TGFα and TGFβ_1 expression in normal-appearing colorectal mucosa predicts adenoma recurrence and colorectal cancer risk in a large of cohort of colorectal adenoma patients after adenoma removal. Also, the molecular basis of colorectal carcinogenesis is becoming clearer (371), aiding in the development of pre-neoplastic biomarkers of risk for CRC. I propose to investigate whether other hypothesis-based biomarkers in normal-appearing colorectal mucosa predict adenoma recurrence and colorectal cancer risk. The candidate tissue biomarkers could be MSH2, MLH1, APC, β-catenin, KRAS, BRAF, PIK3CA, PTEN, TP53, BAX, SMAD4, TGFBR2, COX-2, 15-PGDH, and EGFR. These tissue markers would be measured using mQD-IHC/qIA that allows detection and analysis of the amounts, distributions within the gastric epithelium architecture, and relationships to one another.

Finally, based on the findings from the pilot, randomized, double-blind, placebo-controlled, 2 x 2 factorial clinical trial that supplemental calcium and vitamin D increased

$TGF\beta_1$ expression and shifted TGFα expression downward into proliferation zone and decreases in the mean ratio of rectal TGFα to $TGF\beta_1$ expression, I propose to investigate whether the changes of TGFα and $TGF\beta_1$ expression by calcium and vitamin D are associated with the risk of recurrent adenoma and colorectal cancer in a larger randomized, double-blind, placebo-controlled, 2 x 2 factorial clinical trial of calcium and/or vitamin D_3 to further validate whether TGFα and $TGF\beta_1$ expression could serve as surrogate endpoints in chemoprevention trials to assess the potential efficacy, safety, and optimal dose of interventions.

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Appendix

Table A5.1. Selected characteristics of incident, sporadic colorectal adenoma cases and healthy controls among those with usable biomarker measurement.

Characteristics [a]	TGFα sample			$TGFβ_1$ sample			TGFβRII sample		
	Cases (n=35)	Controls (n=36)	P-value [b]	Cases (n=35)	Controls (n=36)	P-value [b]	Cases (n=35)	Controls (n=36)	P-value [b]
Demographics, medical history, habits, anthropometrics									
Age (yrs.)	56.1 ± 6.7	54.8 ± 8.3	0.50	56.0 ± 6.6	56.1 ± 8.5	0.94	56.7 ± 6.0	52.9 ± 7.7	0.11
Male (%)	51.4	47.2	0.81	51.2	55.8	0.83	38.9	52.6	0.51
Caucasian (%)	97.1	97.2	1.00	97.7	97.7	1.00	100	100	1.00
1º relative with CRC (%)	12.1	23.5	0.34	12.2	19.5	0.55	5.9	23.5	0.34
Take NSAID ≥1 week (%)	34.3	52.8	0.15	34.9	41.9	0.66	33.3	73.7	0.02
Take Aspirin ≥1 week (%)	31.4	41.7	0.46	37.2	41.9	0.83	38.9	26.3	0.50
Use HRT (females) (%)	93.3	66.7	0.10	89.5	77.8	0.40	100	66.7	0.21
Current smoker (%)	22.9	8.3	0.11	25.6	4.7	0.01	33.3	0	0.01
Consume alcohol currently (%)	71.4	63.9	0.61	65.1	62.8	1.00	61.1	63.2	1.00
Body mass index (kg/m2)	31.3 ± 8.0	32.1 ± 7.4	0.66	31.1 ± 7.4	31.1 ± 7.2	0.99	32.8 ± 8.3	34.3 ± 7.7	0.57
Waist-to-hip ratio	0.94 ± 0.10	0.92 ± 0.17	0.67	0.93 ± 0.10	0.94 ± 0.15	0.95	0.94 ± 0.10	0.94 ± 0.20	0.98
Total physical activity (METs/day)	51.3 ± 16.0	46.0 ± 11.0	0.11	50.5 ± 14.8	48.5 ± 12.8	0.50	53.4 ± 19.0	49.6 ± 14.7	0.50
Serum 25(OH)D3, ng/mL	25.0 ± 10.8	28.8 ± 13.8	0.26	27.2 ± 12.3	28.6 ± 12.2	0.63	25.0 ± 10.9	29.9 ± 10.3	0.27
Dietary intakes [c]									
Total energy (kcal/day)	2,034 ± 778	1,623 ± 392	<0.01	1,957 ± 763	1,565 ± 421	<0.01	1,809 ± 909	1,705 ± 395	0.66
Total fat (g/day)	65.2 ±	65.6 ± 16.5	0.91	66.6 ± 16.4	67.0 ± 14.9	0.88	64.5 ±	67.3 ±	0.58

	17.1						16.6	13.0	
Saturated fat (g/day)	21.6 ± 6.1	22.1 ± 5.7	0.73	22.0 ± 5.9	22.1 ± 5.2	0.92	21.4 ± 5.2	22.2 ± 4.9	0.62
Total folate (µg/day) [d]	481.5 ± 232.1	496.5 ± 273.9	0.80	463.6 ± 224.2	541.0 ± 284.1	0.16	458.7 ± 209.0	510.5 ± 300.1	0.55
Total fiber (g/day) [d]	15.2 ± 5.8	15.7 ± 6.5	0.72	15.0 ± 5.5	15.6 ± 6.0	0.61	13.8 ± 5.0	15.9 ± 7.7	0.34
Total calcium intake (mg/day) [d]	922.1 ± 524.3	925.6 ± 481.4	0.98	918.8 ± 495.8	988.6 ± 495.6	0.52	961.5 ± 575.7	965.1 ± 488.7	0.98
Total vitamin D intake (IU/day) [d]	326.5 ± 299.7	305.1 ± 271.0	0.75	327.4 ± 291.3	385.3 ± 286.7	0.36	308.0 ± 263.0	319.6 ± 291.0	0.90
Fruits and vegetables (servings/wk.)	29.9 ± 16.2	26.5 ± 11.5	0.31	29.5 ± 16.0	25.0 ± 11.2	0.13	25.3 ± 17.0	27.1 ± 10.7	0.70
Red meat (servings/wk.)	6.3 ± 3.9	4.9 ± 2.7	0.10	6.1 ± 4.0	5.6 ± 5.7	0.61	6.1 ± 3.9	5.9 ± 3.5	0.90
Processed meat (servings/wk.)	2.6 ± 2.3	2.3 ± 1.7	0.55	2.8 ± 2.6	2.4 ± 1.8	0.34	2.4 ± 2.3	2.8 ± 2.2	0.58

[a] Table reports % for categorical variables and mean ± standard deviation for continuous variables

[b] From Fisher's exact test for categorical variables and t test for continuous variables

[c] Energy adjusted using residual method

[d] Total values include diet and supplements

Abbreviations: CRC, colorectal cancer; HRT, hormone replacement therapy; NSAID, nonsteroidal anti-inflammatory drug; MET, metabolic equivalents

Table A5.2. TGFβ_1, TGFα, and TGFα/TGFβ_1 expression ratio in normal-appearing sigmoid mucosa, by case-control status [a]

Marker	Mean	SE	Mean	SE	% diff [b]	P-value [c]	OR (95% CI)	Model covariates
TGFβ_1	**Cases (N = 18)**		**Controls (N = 39)**					
	1.08	0.12	1.11	0.08	-2.23	0.87	1.18 (0.38 - 3.68)	Age and sex
	1.17	0.14	1.15	0.10	1.64	0.69	1.42 (0.42 - 4.84)	Age, sex, and family hx CRC
	1.13	0.13	1.08	0.08	4.61	0.74	1.82 (0.52 - 6.41)	Age, sex, and energy intake
	1.09	0.13	1.11	0.08	-2.03	0.88	1.19 (0.38 - 3.73)	Age, sex, and NSAID use
	1.10	0.13	1.14	0.12	-3.14	0.82	1.21 (0.38 - 3.86)	Age, sex, and smoking
TGFα	**Cases (N = 9)**		**Controls (N = 10)**					
	1.15	0.21	0.95	0.20	20.81	0.51	1.25 (0.18 - 8.62)	Age and sex
	1.04	0.28	0.84	0.24	24.79	0.56	0.75 (0.08 - 7.41)	Age, sex, and family hx CRC
	1.18	0.21	0.92	0.20	27.72	0.41	1.09 (0.15 - 7.97)	Age, sex, and energy intake
	1.04	0.23	0.95	0.20	9.51	0.77	0.80 (0.09 - 7.05)	Age, sex, and NSAID use
	1.20	0.36	1.02	0.42	17.64	0.58	1.06 (0.15 - 7.55)	Age, sex, and smoking
TGFα/TGFβ_1	**Cases (N = 9)**		**Controls (N = 10)**					
ratio	1.01	0.22	0.94	0.21	6.52	0.84	1.39 (0.21 - 9.26)	Age and sex
	0.87	0.30	0.92	0.26	-5.69	0.89	0.91 (0.10 - 8.07)	Age, sex, and family hx CRC
	1.03	0.22	0.92	0.21	11.88	0.73	1.32 (0.19 - 9.05)	Age, sex, and energy intake
	0.86	0.22	0.94	0.19	-8.72	0.78	0.86 (0.10 - 7.40)	Age, sex, and NSAID use
	1.43	0.33	1.49	0.39	-4.27	0.83	1.15 (0.17 - 7.95)	Age, sex, and smoking

[a] Marker expression quantified by batched-standardized optical density values

[b] % diff = proportional difference, calculated as {(mean in cases- mean in controls) ÷ mean in controls} *100%

[c] p-value for difference by ANACOVA

Abbreviations: SE, standard error; hx CRC, history of colorectal cancer (in a first degree relative); NSAID, nonsteroidal anti-inflammatory drug (≥ once/wk.)

Table SA.3. $TGF\beta_1$, $TGF\alpha$, and $TGF\alpha/TGF\beta_1$ expression ratio in normal-appearing ascending colon mucosa, by case-control status [a]

Marker	Mean	SE	Mean	SE	% diff [b]	P-value [c]	OR (95% CI)	Model covariates
$TGF\beta_1$	**Cases (N = 16)**		**Controls (N = 44)**					
	0.92	0.12	0.95	0.07	-3.17	0.82	0.35 (0.09 - 1.29)	Age and sex
	0.93	0.13	0.94	0.09	-0.28	0.99	0.48 (0.13 - 1.83)	Age, sex, and family hx CRC
	0.91	0.12	0.96	0.07	-4.23	0.77	0.34 (0.09 - 1.29)	Age, sex, and energy intake
	0.93	0.12	0.96	0.07	-2.48	0.86	0.35 (0.10 - 1.31)	Age, sex, and NSAID use
	0.83	0.12	0.81	0.09	2.72	0.87	0.39 (0.10 - 1.49)	Age, sex, and smoking
$TGF\alpha$	**Cases (N = 9)**		**Controls (N = 10)**					
	0.92	0.38	1.30	0.36	-29.33	0.48	0.36 (0.04 - 2.94)	Age and sex
	0.90	0.61	1.37	0.44	-33.97	0.48	0.54 (0.06 - 5.37)	Age, sex, and family hx CRC
	0.93	0.40	1.29	0.37	-27.98	0.53	0.42 (0.05 - 3.58)	Age, sex, and energy intake
	0.72	0.41	1.24	0.36	-41.86	0.35	0.27 (0.03 - 2.56)	Age, sex, and NSAID use
	0.45	0.51	0.83	0.49	-45.35	0.47	0.25 (0.02 - 3.24)	Age, sex, and smoking
$TGF\alpha/TGF\beta_1$	**Cases (N = 9)**		**Controls (N = 10)**					
ratio	1.26	0.37	1.18	0.35	7.22	0.87	0.78 (0.12 - 5.26)	Age and sex
	1.27	0.60	1.26	0.44	0.88	0.99	2.11 (0.19 - 23.07)	Age, sex, and family hx CRC
	1.32	0.38	1.13	0.36	16.97	0.72	0.80 (0.12 - 5.60)	Age, sex, and energy intake
	1.18	0.42	1.15	0.36	1.94	0.97	0.64 (0.09 - 4.70)	Age, sex, and NSAID use
	1.67	0.50	1.59	0.49	5.22	0.87	0.78 (0.11 - 5.29)	Age, sex, and smoking

[a] Marker expression quantified by batched-standardized optical density values

[b] % diff = proportional difference, calculated as {(mean in cases- mean in controls) ÷ mean in controls} *100%

[c] p-value for difference by ANACOVA

Abbreviations: SE, standard error; hx CRC, history of colorectal cancer (in a first degree relative); NSAID, nonsteroidal anti-inflammatory drug (≥ once/wk.)

Table A5.4. TGFα, TGFβ_1, and TGFα/TGFβ_1 expression ratio in rectum normal-looking mucosa samples by potential risk factors for colorectal neoplasms

	TGFα/TGFβ$_1$ ratio (Cases/controls: 35/29)					**TGFα** (Cases/controls: 35/36)					**TGFβ$_1$** (Cases/controls: 43/43)				
Risk Factors	N	Mean	SE	% diff[a]	p-value[b]	N	Mean	SE	% diff[a]	p-value[b]	N	Mean	SE	% diff[a]	p-value[b]
Age, yrs															
<55	35	1.62	0.36	16.69	0.63	41	0.98	0.13	2.41	0.91	47	0.97	0.16	12.73	0.61
≥55	29	1.89	0.39			30	1.00	0.15			39	1.09	0.17		
Sex															
Female	30	1.76	0.38	-0.34	0.99	36	1.03	0.14	-6.98	0.73	40	1.02	0.17	0.51	0.98
Male	34	1.75	0.37			35	0.96	0.15			46	1.03	0.16		
Family hx of CRC															
No	49	1.74	0.30	2.28	0.96	55	0.92	0.11	24.50	0.40	69	1.03	0.13	-11.40	0.72
Yes	11	1.78	0.64			12	1.15	0.24			13	0.91	0.30		
NSAID use															
No	37	1.46	0.33	48.05	0.18	40	0.95	0.13	9.37	0.65	53	1.01	0.14	5.53	0.81
Yes	27	2.17	0.39			31	1.04	0.15			33	1.06	0.18		
Aspririn use															
No	42	1.83	0.32	-12.21	0.68	45	1.07	0.12	-19.18	0.31	52	0.97	0.15	15.33	0.53
Yes	22	1.61	0.44			26	0.86	0.16			34	1.12	0.18		
Smoking status															
Never/former	55	1.53	0.27	104.53	0.03	60	0.99	0.11	-1.71	0.95	73	1.12	0.12	-52.25	0.06
Current	9	3.12	0.66			11	0.98	0.25			13	0.53	0.28		
Drinking status															
Never/former	20	1.96	0.47	-15.16	0.61	23	1.09	0.18	-12.86	0.52	31	1.07	0.19	-5.98	0.79
Current	44	1.66	0.32			48	0.95	0.12			55	1.00	0.14		
Body mass index, kg/m^2															
<30	30	1.26	0.36	74.57	0.07	36	1.06	0.14	-13.70	0.46	44	1.04	0.16	-2.49	0.91
≥30	34	2.20	0.34			35	0.92	0.14			42	1.01	0.16		
Waist-to-hip ratio															
Low	23	1.61	0.43	14.44	0.67	27	1.09	0.16	-14.34	0.44	32	0.86	0.19	30.39	0.26
High	41	1.84	0.32			44	0.93	0.12			54	1.12	0.14		
Physical activity, METs/d															

Low	32	1.61	0.36	17.50	0.59	38	1.06	0.13	-12.93	0.48	44	1.16	0.16	-23.78	0.22
High	32	1.89	0.36			33	0.92	0.14			42	0.89	0.16		
Serum 25(OH)D_3, ng/mL															
Low	29	1.84	0.40	-19.67	0.56	31	1.15	0.17	-20.64	0.50	39	1.10	0.17	-22.13	0.33
High	19	1.48	0.47			22	0.92	0.19			31	0.86	0.19		
Total energy intake[c]															
Low	23	1.63	0.43	11.82	0.72	26	1.03	0.16	-5.64	0.77	34	1.11	0.18	-12.04	0.57
High	41	1.82	0.32			45	0.97	0.12			52	0.97	0.15		
Total fat[c]															
Low	31	1.88	0.37	-13.57	0.63	37	1.06	0.14	-13.86	0.47	40	0.83	0.16	44.76	0.12
High	33	1.63	0.37			34	0.91	0.14			46	1.20	0.15		
Saturated fat[c]															
Low	29	1.82	0.38	-6.84	0.81	33	1.18	0.14	-30.00	0.07	41	0.84	0.16	43.77	0.10
High	35	1.70	0.35			38	0.82	0.13			45	1.20	0.15		
Total folate[c]															
Low	32	1.90	0.37	-14.85	0.59	37	0.92	0.14	16.31	0.46	44	1.17	0.16	-24.58	0.21
High	32	1.62	0.36			34	1.07	0.14			42	0.88	0.16		
Total fiber[c]															
Low	33	1.75	0.36	0.90	0.98	38	0.98	0.14	2.43	0.90	42	1.05	0.16	-4.23	0.85
High	31	1.76	0.37			33	1.00	0.14			44	1.01	0.16		
Total calcium intake[c]															
Low	34	1.90	0.35	-16.37	0.55	39	1.03	0.13	-7.36	0.70	42	0.90	0.16	27.76	0.28
High	30	1.59	0.37			32	0.95	0.14			44	1.15	0.16		
Total vitamin D intake[c]															
Low	35	1.81	0.35	-6.93	0.81	40	0.92	0.13	16.12	0.46	42	1.01	0.16	2.93	0.90
High	29	1.69	0.38			31	1.07	0.15			44	1.04	0.16		
Fruit and vegetables[c]															
Low	31	1.53	0.37	27.71	0.41	35	1.08	0.14	-15.79	0.39	42	1.19	0.16	-27.27	0.15
High	33	1.96	0.35			36	0.91	0.14			44	0.87	0.16		
Red meat[c]															
Low	26	2.16	0.40	-31.27	0.20	29	1.16	0.15	-	0.16	39	1.02	0.17	0.53	0.98

High	38	1.48	0.33			42	0.88	0.13	24.04		47	1.03	0.15		
Processed meat[c]															
Low	25	2.36	0.40	-41.91	0.06	30	1.01	0.16	-2.83	0.89	38	0.94	0.17	17.06	0.49
High	39	1.37	0.32			41	0.98	0.13			48	1.10	0.15		

[a] Proportional difference = [(mean of comparative category - mean of referent category)/mean of referent category] *100%.

[b] p-value for difference by ANCOVA, adjusted for age and sex, except for sex and age.

[c] Energy adjusted variables except for energy intake and physical activity; dichotomized based on sex-specific median value in controls

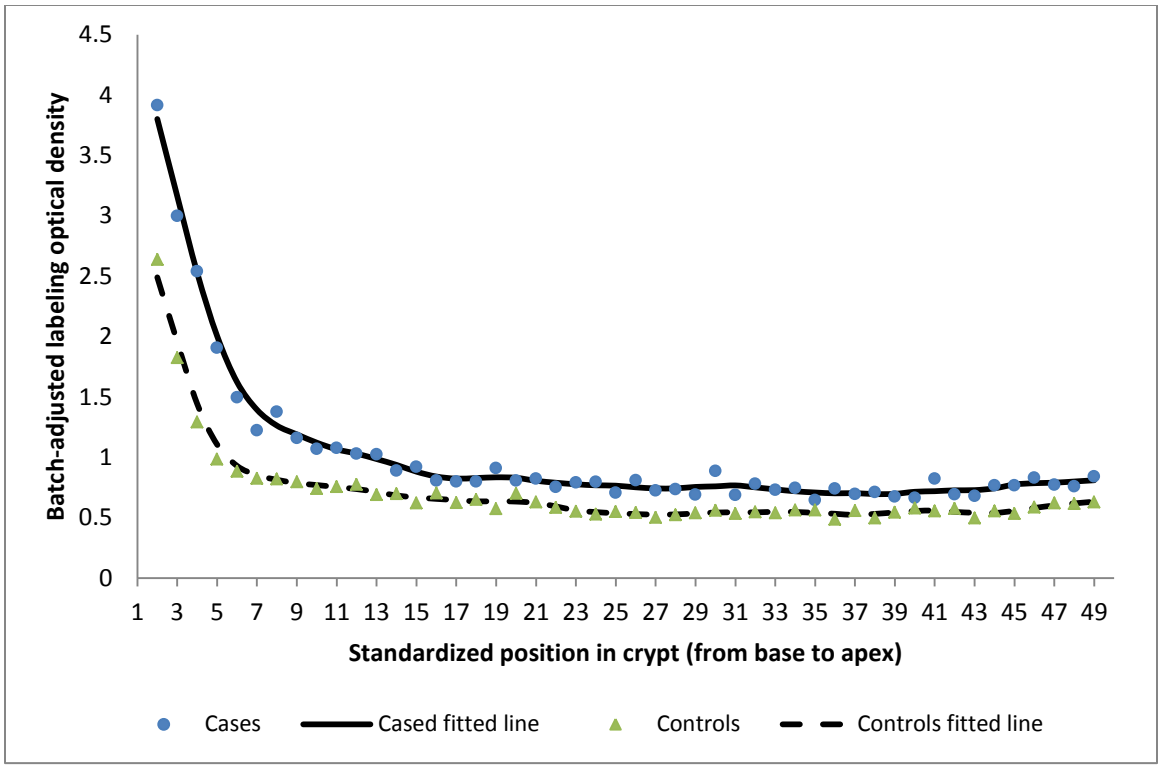

Figure A5.1. Batch-standardized expression (as optical density of biomarker labeling) of TGFα along lengths of normal colorectal crypts by case-control status
Abbreviation: TGFα, transforming growth factor alpha

Table A6.1. Sensitivity analysis of treatment effect on expression of transforming growth factor alpha (TGFα) and transforming growth factor beta 1 ($TGF\beta_1$) in the normal-appearing colorectal mucosa following multiple imputation for missing observations

	Treatment Group	Baseline		6-mo follow-up		Absolute Rx effect		
		n	Mean	n	Mean	Rx effect [a]	Std Err	P [b]
A. TGFα								
Whole crypts								
	Placebo	23	0.96	23	1.00	0.00	.	.
	Calcium	23	1.00	23	0.97	-0.07	0.16	0.67
	Vitamin D_3	23	0.85	23	0.96	0.07	0.16	0.66
	Ca + Vit. D_3	23	0.93	23	1.02	0.06	0.16	0.71
Upper 40% of crypts								
	Placebo	23	0.53	23	0.53	0.00	.	.
	Calcium	23	0.67	23	0.52	-0.15	0.09	0.11
	Vitamin D_3	23	0.55	23	0.53	-0.02	0.09	0.81
	Ca + Vit. D_3	23	0.56	23	0.53	-0.03	0.09	0.71
Lower 60% of crypts								
	Placebo	23	0.42	23	0.47	0.00	.	.
	Calcium	23	0.34	23	0.44	0.05	0.09	0.63
	Vitamin D_3	23	0.29	23	0.46	0.12	0.10	0.21
	Ca + Vit. D_3	23	0.35	23	0.48	0.08	0.09	0.42
B. $TGF\beta_1$								
Whole crypts								
	Placebo	23	1.03	23	0.99	0.00	.	.
	Calcium	23	1.03	23	1.13	0.15	0.12	0.22
	Vitamin D_3	23	0.93	23	1.06	0.18	0.12	0.15
	Ca + Vit. D_3	23	1.02	23	1.18	0.20	0.12	0.09
Upper 40% of crypts								
	Placebo	23	0.39	23	0.38	0.00	.	.
	Calcium	23	0.40	23	0.43	0.04	0.04	0.40
	Vitamin D_3	23	0.35	23	0.41	0.07	0.04	0.14
	Ca + Vit. D_3	23	0.39	23	0.45	0.06	0.04	0.15
Lower 60% of crypts								
	Placebo	23	0.65	23	0.61	0.00	.	.
	Calcium	23	0.64	23	0.71	0.10	0.07	0.15
	Vitamin D_3	23	0.58	23	0.67	0.12	0.07	0.08

	Ca + Vit. D_3	23	0.60	23	0.70	0.13	0.07	0.06

[a] Rx effect = [(treatment group follow-up) − (treatment group baseline)] − [(placebo group follow-up) − (placebo group baseline)].

[b] P value for difference between each active treatment group and placebo group from repeated-measures MIXED model.

Abbreviation: TGFα, transforming growth factor alpha; TGFβ_1 transforming growth factor beta 1

Table A6.2. Standardized expression of transforming growth factor alpha (TGFα) and transforming growth factor beta 1 ($TGF\beta_1$) in the normal-appearing colorectal mucosa during the clinical trial.

	Baseline				6-mo follow-up				Absolute Rx effect				
Treatment Group	n	Mean	Std Err	P	n	Mean	Std Err	P	n	Rx effect [1]	Std Err	P [2]	Relative Effect [3]
A. TGFα													
Control for baseline NSAIDs use													
Placebo	20	1.06	0.13		21	1.00	0.04		18	0	.	.	1.00
Calcium	23	1.00	0.08	0.71	21	0.97	0.08	0.74	21	0.03	0.16	0.87	1.03
Vitamin D_3	20	0.96	0.09	0.53	21	0.98	0.06	0.85	19	0.09	0.16	0.61	1.09
Ca + Vit. D_3	21	0.98	0.14	0.62	21	1.01	0.07	0.88	19	0.09	0.16	0.59	1.10
Control for baseline serum 25-OH-vitamin D concentrations													
Placebo	20	0.59	0.07		21	0.53	0.02		18	0	.	.	1.00
Calcium	23	0.67	0.06	0.42	21	0.52	0.04	0.79	21	-0.09	0.10	0.35	0.86
Vitamin D_3	20	0.61	0.06	0.84	21	0.54	0.03	0.89	19	-0.01	0.10	0.86	0.98
Ca + Vit. D_3	21	0.59	0.08	0.95	21	0.53	0.03	0.97	19	-0.01	0.10	0.93	0.99
Control for baseline calcium intake													
Placebo	20	0.47	0.08		21	0.47	0.02		18	0	.	.	1.00
Calcium	23	0.34	0.04	0.13	21	0.44	0.04	0.53	21	0.10	0.10	0.30	1.29
Vitamin D_3	20	0.35	0.06	0.18	21	0.48	0.04	0.82	19	0.13	0.10	0.17	1.38
Ca + Vit. D_3	21	0.39	0.07	0.34	21	0.48	0.04	0.83	19	0.10	0.10	0.32	1.25
B. $TGF\beta_1$													
Control for NSAIDs use													
Placebo	21	1.04	0.06		21	1.00	0.02		19	0	.	.	1.00
Calcium	22	1.03	0.06	0.95	21	1.13	0.07	0.12	20	0.14	0.13	0.25	1.14
Vitamin D_3	22	0.93	0.07	0.23	20	1.07	0.06	0.42	19	0.17	0.13	0.17	1.19
Ca + Vit. D_3	21	1.00	0.05	0.64	21	1.18	0.07	0.04	19	0.22	0.13	0.09	1.22
Control for baseline serum 25-OH-vitamin D concentrations													
Placebo	21	0.39	0.02		21	0.38	0.01		19	0	.	.	1.00
Calcium	22	0.40	0.02	0.83	21	0.43	0.03	0.11	20	0.04	0.05	0.35	1.11
Vitamin D_3	22	0.35	0.03	0.25	20	0.41	0.03	0.34	19	0.07	0.05	0.15	1.20
Ca + Vit. D_3	21	0.39	0.02	0.86	21	0.45	0.02	0.03	19	0.08	0.05	0.12	1.20
Control for baseline calcium intake													
Placebo	21	0.65	0.04		21	0.62	0.02		19	0	.	.	1.00
Calcium	22	0.64	0.04	0.80	21	0.70	0.03	0.10	20	0.10	0.07	0.18	1.16
Vitamin D_3	22	0.58	0.04	0.18	20	0.67	0.04	0.37	19	0.11	0.07	0.12	1.20
Ca + Vit. D_3	21	0.59	0.03	0.23	21	0.70	0.04	0.11	19	0.14	0.07	0.06	1.25

[1] Rx effect = [(treatment group follow-up) − (treatment group baseline)] − [(placebo group follow-up) − (placebo group baseline)].

[2] P value for difference between each active treatment group and placebo group from repeated-measures MIXED model.

[3] Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo follow-up) / (placebo baseline)]; interpretation similar to that for an odds ratio (e.g., a relative effect of 1.7 indicates a 70% proportional increase in the treatment group relative to that in the placebo group).

Table A6.3. **Expression of transforming growth factor alpha (TGF α) in the normal-appearing colorectal mucosa during the clinical trial stratified by baseline characteristics.**

	Baseline				6-mo follow-up				Absolute Rx effect				
Treatment Group	***n***	**Mean**	**Std Err**	***P***	***n***	**Mean**	**Std Err**	***P***	***n***	**Rx effect [a]**	**Std Err**	***P* [b]**	**Relative Effect [c]**
Men													
Placebo	15	1.04	0.16		14	0.96	0.04			0	.	.	1.00
Calcium	16	0.99	0.10	0.77	14	0.91	0.10	0.67		0.01	0.20	0.96	1.01
Vitamin D	14	1.05	0.08	0.97	16	0.95	0.06	0.95		-0.01	0.20	0.96	0.99
Ca + Vit D	14	0.90	0.15	0.45	14	0.96	0.06	0.94		0.15	0.20	0.47	1.16
Women													
Placebo	5	1.11	0.27		7	1.09	0.09			0	.	.	1.00
Calcium	7	1.03	0.10	0.82	7	1.08	0.12	0.99		0.08	0.29	0.81	1.08
Vitamin D	6	0.74	0.24	0.31	5	1.09	0.14	0.99		0.37	0.31	0.31	1.50
Ca + Vit D	7	1.14	0.30	0.94	7	1.11	0.15	0.89		0.00	0.29	0.97	1.00
Old (>=59 yrs)													
Placebo	8	0.99	0.21		8	1.10	0.08			0	.	.	1.00
Calcium	13	1.03	0.09	0.87	11	0.92	0.10	0.20		-0.22	0.2442	0.3604	0.80
Vitamin D	10	1.00	0.16	0.95	10	1.06	0.06	0.77		-0.06	0.2537	0.7556	0.95
Ca + Vit D	12	1.06	0.22	0.77	11	1.07	0.11	0.84		-0.10	0.25	0.67	0.90
Young (<59 yrs)													
Placebo	12	1.11	0.18		13	0.94	0.04			0	.	.	1.00
Calcium	10	0.97	0.14	0.50	10	1.03	0.12	0.42		0.23	0.23	0.31	1.26
Vitamin D	10	0.91	0.10	0.34	11	0.92	0.09	0.84		0.18	0.22	0.44	1.19
Ca + Vit D	9	0.87	0.15	0.27	10	0.95	0.07	0.92		0.25	0.23	0.29	1.29
High BMI													
Placebo	12	1.06	0.13		11	1.04	0.07			0	.	.	1.00
Calcium	10	0.99	0.09	0.78	9	0.95	0.13	0.51		-0.03	0.2099	0.8006	0.97
Vitamin D	7	0.87	0.10	0.46	8	0.98	0.08	0.68		0.13	0.225	0.5893	1.14
Ca + Vit D	13	1.14	0.21	0.71	12	0.99	0.09	0.73		-0.12	0.1971	0.4426	0.89
Low BMI													
Placebo	8	1.07	0.29		10	0.96	0.03			0	.	.	1.00
Calcium	13	1.01	0.12	0.81	12	0.99	0.09	0.80		0.09	0.25	0.72	1.09
Vitamin D	13	1.00	0.13	0.79	13	0.99	0.08	0.81		0.09	0.25	0.72	1.09
Ca + Vit D	8	0.73	0.10	0.20	9	1.04	0.10	0.52		0.42	0.27	0.13	1.58
High calcium intake													
Placebo	10	1.30	0.24		11	1.07	0.06			0	.	.	1.00
Calcium	12	0.97	0.10	0.16	12	0.89	0.08	0.06		0.14	0.21	0.52	1.11

Vitamin D	13	0.98	0.13	0.16	12	0.98	0.06	0.34	0.23	0.21	0.33	1.21
Ca + Vit D	8	1.01	0.19	0.26	8	1.02	0.09	0.63	0.24	0.24	0.33	1.23
Low calcium intake												
Placebo	10	0.82	0.09		10	0.92	0.05		0	.	.	1.00
Calcium	10	1.00	0.12	0.44	9	1.08	0.14	0.28	-0.01	0.25	0.92	0.97
Vitamin D	7	0.92	0.12	0.69	9	0.99	0.11	0.64	-0.03	0.27	0.93	0.96
Ca + Vit D	12	0.99	0.21	0.44	13	1.01	0.10	0.52	-0.08	0.24	0.73	0.91
High serum vitamin D												
Placebo	7	1.12	0.15		8	1.02	0.03		0	.	.	1.00
Calcium	16	1.00	0.10	0.57	14	0.99	0.10	0.79	0.09	0.23	0.72	1.08
Vitamin D	11	0.92	0.13	0.37	11	0.99	0.07	0.77	0.16	0.24	0.51	1.17
Ca + Vit D	10	0.79	0.19	0.15	9	1.02	0.10	0.98	0.33	0.25	0.21	1.41
Low serum vitamin D												
Placebo	13	1.03	0.19		13	0.99	0.07		0	.	.	1.00
Calcium	7	1.01	0.13	0.95	7	0.93	0.13	0.71	-0.04	0.26	0.89	0.96
Vitamin D	8	1.10	0.12	0.80	10	0.98	0.09	0.97	-0.07	0.24	0.83	0.94
Ca + Vit D	11	1.16	0.20	0.60	12	1.01	0.09	0.85	-0.10	0.22	0.65	0.91
High fiber intake												
Placebo	11	1.10	0.22		10	1.02	0.08		0	.	.	1.00
Calcium	13	1.06	0.09	0.87	13	0.98	0.10	0.73	0.00	0.22	0.99	0.99
Vitamin D	11	0.96	0.14	0.56	10	0.94	0.05	0.50	0.05	0.23	0.85	1.05
Ca + Vit D	9	0.80	0.21	0.23	9	0.95	0.08	0.58	0.23	0.24	0.34	1.28
Low fiber intake												
Placebo	9	1.01	0.14		11	0.98	0.03		0	.	.	1.00
Calcium	9	0.87	0.13	0.54	8	0.96	0.12	0.86	0.12	0.25	0.67	1.14
Vitamin D	9	0.95	0.13	0.80	11	1.03	0.10	0.73	0.10	0.24	0.65	1.11
Ca + Vit D	11	1.16	0.20	0.51	12	1.06	0.10	0.52	-0.07	0.23	0.77	0.95
High alcohol consumption												
Placebo	10	0.83	0.10		10	1.00	0.03		0	.	.	1.00
Calcium	13	1.00	0.11	0.38	13	1.00	0.10	1.00	-0.17	0.20	0.40	0.83
Vitamin D	12	0.87	0.10	0.82	12	1.06	0.08	0.67	0.01	0.20	0.97	1.00
Ca + Vit D	8	1.11	0.26	0.21	8	1.20	0.11	0.16	-0.08	0.23	0.72	0.89
Low alcohol consumption												
Placebo	10	1.29	0.23		11	1.00	0.08		0	.	.	1.00
Calcium	9	0.96	0.12	0.22	8	0.92	0.12	0.51	0.25	0.26	0.35	1.24
Vitamin D	8	1.08	0.17	0.45	9	0.88	0.07	0.35	0.10	0.26	0.72	1.06
Ca + Vit D	12	0.92	0.18	0.15	13	0.90	0.07	0.37	0.27	0.23	0.24	1.26
Family history of colorectal cancer or polyp												
Placebo	10	1.09	0.21		10	0.97	0.02		0	.	.	1.00

Calcium	13	0.90	0.11	0.43	11	0.92	0.09	0.66	0.14	0.24	0.57	1.15
Vitamin D	7	0.93	0.19	0.56	7	0.97	0.10	0.99	0.17	0.28	0.61	1.18
Ca + Vit D	4	0.67	0.40	0.22	4	0.89	0.16	0.58	0.34	0.33	0.32	1.49
No family history of colorectal cancer or polyp												
Placebo	10	1.03	0.18		11	1.03	0.08		0	.	.	1.00
Calcium	10	1.13	0.09	0.64	10	1.03	0.12	0.99	-0.11	0.22	0.64	0.91
Vitamin D	11	0.96	0.10	0.77	12	0.95	0.07	0.55	-0.01	0.22	1.00	0.99
Ca + Vit D	16	1.06	0.16	0.88	16	1.05	0.08	0.82	0.00	0.20	0.99	1.00
Regular use of NSAID or Aspirin												
Placebo	10	0.89	0.10		10	0.97	0.06		0	.	.	1.00
Calcium	13	1.03	0.09	0.52	11	1.09	0.11	0.38	-0.03	0.22	0.90	0.97
Vitamin D	6	1.06	0.12	0.53	6	0.88	0.06	0.53	-0.26	0.26	0.33	0.76
Ca + Vit D	14	1.09	0.20	0.35	13	1.07	0.09	0.42	-0.10	0.21	0.62	0.90
No regular use of NSAID or Aspirin												
Placebo	10	1.23	0.24		11	1.02	0.06		0	.	.	1.00
Calcium	10	0.96	0.13	0.27	10	0.84	0.09	0.12	0.08	0.24	0.73	1.05
Vitamin D	14	0.92	0.12	0.16	15	1.03	0.07	0.98	0.32	0.22	0.16	1.34
Ca + Vit D	7	0.77	0.12	0.08	8	0.92	0.10	0.38	0.35	0.26	0.18	1.43

[a] Rx effect = [(treatment group follow-up) − (treatment group baseline)] − [(placebo group follow-up) − (placebo group baseline)].

[b] P value for difference between each active treatment group and placebo group from repeated-measures MIXED model.

[c] Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo follow-up) / (placebo baseline)]; interpretation similar to that for an odds ratio (e.g., a relative effect of 1.7 indicates a 70% proportional increase in the treatment group relative to that in the placebo group).

Table A.6.4. Expression of transforming growth factor beta 1 (TGFβ_1) in the normal-appearing colorectal mucosa during the clinical trial stratified by baseline characteristics.

	Baseline				6-mo follow-up				Absolute Rx effect				
Treatment Group	*n*	Mean	Std Err	*P*	*n*	Mean	Std Err	*P*	*n*	Rx effect*	Std Err	*P***	Relative Effect¥
Men													
Placebo	14	1.08	0.07		14	0.99	0.03			0	.	.	1.00
Calcium	15	1.10	0.07	0.83	14	1.11	0.09	0.24		0.10	0.14	0.47	1.10
Vitamin D	15	1.03	0.06	0.56	14	0.99	0.07	0.99		0.05	0.14	0.69	1.05
Ca + Vit D	14	1.01	0.07	0.43	14	1.18	0.08	0.06		0.27	0.14	0.06	1.28
Women													
Placebo	7	0.95	0.11		7	1.01	0.04			0	.	.	1.00
Calcium	7	0.88	0.10	0.68	7	1.17	0.10	0.31		0.22	0.23	0.35	1.24
Vitamin D	7	0.73	0.16	0.20	6	1.24	0.11	0.15		0.45	0.24	0.07	1.59
Ca + Vit D	7	0.98	0.09	0.85	7	1.16	0.15	0.33		0.12	0.23	0.62	1.11
Old (>=59 yrs)													
Placebo	9	1.05	0.08		8	1.01	0.03			0	.	.	1.00
Calcium	12	1.10	0.08	0.69	11	1.17	0.10	0.19		0.12	0.19	0.51	1.11
Vitamin D	12	0.93	0.11	0.34	10	1.19	0.09	0.17		0.31	0.20	0.12	1.34
Ca + Vit D	12	0.96	0.07	0.50	11	1.23	0.08	0.09		0.31	0.19	0.12	1.33
Young (<59 yrs)													
Placebo	12	1.03	0.09		13	1.00	0.03			0	.	.	1.00
Calcium	10	0.95	0.08	0.50	10	1.09	0.08	0.42		0.17	0.17	0.31	1.18
Vitamin D	10	0.94	0.08	0.47	10	0.95	0.08	0.67		0.04	0.17	0.80	1.04
Ca + Vit D	9	1.04	0.09	0.89	10	1.12	0.12	0.26		0.11	0.17	0.53	1.11
High BMI													
Placebo	13	1.01	0.08		11	0.99	0.04			0	.	.	1.00
Calcium	10	0.94	0.07	0.60	9	1.19	0.12	0.17		0.27	0.21	0.18	1.29
Vitamin D	9	0.94	0.14	0.61	9	1.06	0.12	0.65		0.13	0.21	0.49	1.14
Ca + Vit D	13	1.06	0.08	0.67	12	1.21	0.11	0.11		0.16	0.19	0.38	1.16
Low BMI													
Placebo	8	1.09	0.09		10	1.01	0.03			0	.	.	1.00
Calcium	12	1.11	0.08	0.84	12	1.09	0.08	0.42		0.06	0.15	0.70	1.06
Vitamin D	13	0.93	0.07	0.16	11	1.08	0.07	0.50		0.23	0.15	0.13	1.25
Ca + Vit D	8	0.90	0.04	0.13	9	1.14	0.09	0.23		0.32	0.16	0.05	1.37
High calcium intake													
Placebo	10	1.03	0.08		11	1.04	0.02			0	.	.	1.00
Calcium	12	1.17	0.08	0.27	12	1.05	0.07	0.88		-0.12	0.17	0.46	0.89

Vitamin D	14	0.91	0.09	0.34	11	1.14	0.08	0.33	0.22	0.17	0.19	1.24
Ca + Vit D	8	1.09	0.08	0.69	8	1.32	0.12	0.02	0.22	0.19	0.24	1.20
Low calcium intake												
Placebo	11	1.05	0.09		10	0.96	0.04		0	.	.	1.00
Calcium	9	0.88	0.06	0.16	9	1.24	0.12	0.04	0.45	0.18	0.02	1.53
Vitamin D	8	0.97	0.10	0.55	9	0.98	0.10	0.88	0.09	0.19	0.58	1.10
Ca + Vit D	12	0.96	0.07	0.45	13	1.09	0.08	0.27	0.21	0.17	0.21	1.23
High serum vitamin D												
Placebo	8	1.08	0.11		8	1.01	0.02		0	.	.	1.00
Calcium	16	1.02	0.07	0.57	14	1.14	0.09	0.26	0.19	0.16	0.23	1.20
Vitamin D	11	0.97	0.08	0.32	10	1.13	0.07	0.32	0.24	0.17	0.16	1.26
Ca + Vit D	10	0.87	0.04	0.07	9	1.16	0.09	0.24	0.37	0.18	0.04	1.44
Low serum vitamin D												
Placebo	13	1.01	0.07		13	0.99	0.03		0	.	.	1.00
Calcium	6	1.06	0.12	0.74	7	1.12	0.10	0.38	0.07	0.22	0.73	1.07
Vitamin D	10	0.92	0.13	0.49	10	1.00	0.11	0.95	0.10	0.19	0.59	1.11
Ca + Vit D	11	1.12	0.08	0.40	12	1.19	0.11	0.10	0.09	0.19	0.67	1.08
High fiber intake												
Placebo	11	1.06	0.08		10	0.99	0.04		0	.	.	1.00
Calcium	12	1.15	0.08	0.45	13	1.16	0.10	0.16	0.07	0.18	0.70	1.07
Vitamin D	12	0.88	0.11	0.17	10	1.18	0.09	0.13	0.36	0.18	0.05	1.43
Ca + Vit D	9	1.07	0.09	0.92	9	1.21	0.08	0.09	0.21	0.19	0.28	1.21
Low fiber intake												
Placebo	10	1.02	0.10		11	1.01	0.02		0	.	.	1.00
Calcium	9	0.90	0.06	0.29	8	1.09	0.06	0.49	0.20	0.18	0.24	1.23
Vitamin D	10	1.00	0.08	0.86	10	0.96	0.08	0.66	-0.03	0.17	0.91	0.97
Ca + Vit D	11	0.97	0.06	0.63	12	1.15	0.11	0.21	0.19	0.17	0.27	1.20
High alcohol consumption												
Placebo	9	1.03	0.06		10	1.01	0.02		0	.	.	1.00
Calcium	13	1.06	0.08	0.80	13	1.15	0.09	0.25	0.11	0.19	0.58	1.10
Vitamin D	13	0.86	0.10	0.16	12	1.14	0.09	0.28	0.31	0.19	0.11	1.37
Ca + Vit D	8	1.05	0.10	0.92	8	1.25	0.11	0.08	0.23	0.21	0.32	1.22
Low alcohol consumption												
Placebo	12	1.04	0.10		11	0.99	0.04		0	.	.	1.00
Calcium	8	1.01	0.10	0.81	8	1.11	0.09	0.34	0.15	0.17	0.41	1.15
Vitamin D	9	1.05	0.08	0.97	8	0.96	0.08	0.78	-0.04	0.17	0.81	0.96
Ca + Vit D	12	0.99	0.06	0.66	13	1.13	0.10	0.20	0.19	0.15	0.23	1.20
Family history of colorectal cancer or polyp												
Placebo	10	1.00	0.11		10	1.02	0.03		0	.	.	1.00

Calcium	13	1.05	0.07	0.67	11	1.07	0.09	0.62	0.00	0.13	0.87	1.00
Vitamin D	8	0.92	0.08	0.59	5	0.99	0.12	0.79	0.04	0.16	0.88	1.04
Ca + Vit D	4	1.01	0.08	0.90	4	1.26	0.14	0.10	0.21	0.18	0.28	1.20
No family history of colorectal cancer or polyp												
Placebo	11	1.08	0.06		11	0.98	0.04		0	.	.	1.00
Calcium	9	1.01	0.11	0.62	10	1.20	0.10	0.09	0.28	0.19	0.15	1.29
Vitamin D	11	0.98	0.09	0.41	12	1.06	0.08	0.53	0.18	0.18	0.33	1.19
Ca + Vit D	16	1.00	0.07	0.47	16	1.16	0.09	0.11	0.26	0.17	0.13	1.28
Regular use of NSAID or Aspirin												
Placebo	9	0.99	0.08		10	1.02	0.04		0	.	.	1.00
Calcium	12	1.08	0.08	0.42	11	1.18	0.09	0.16	0.06	0.18	0.69	1.05
Vitamin D	7	0.85	0.14	0.32	6	1.07	0.13	0.69	0.19	0.21	0.38	1.23
Ca + Vit D	14	0.97	0.06	0.92	13	1.13	0.07	0.28	0.13	0.17	0.48	1.13
No regular use of NSAID or Aspirin												
Placebo	12	1.08	0.09		11	0.99	0.03		0	.	.	1.00
Calcium	10	0.97	0.08	0.39	10	1.08	0.09	0.46	0.20	0.19	0.27	1.22
Vitamin D	15	0.98	0.08	0.37	14	1.07	0.08	0.48	0.18	0.17	0.26	1.20
Ca + Vit D	7	1.04	0.11	0.81	8	1.24	0.16	0.06	0.29	0.20	0.15	1.30

[a] Rx effect = [(treatment group follow-up) − (treatment group baseline)] − [(placebo group follow-up) − (placebo group baseline)].

[b] P value for difference between each active treatment group and placebo group from repeated-measures MIXED model.

[c] Relative effect = [(treatment group follow-up) / (treatment group baseline)] / [(placebo follow-up) / (placebo baseline)]; interpretation similar to that for an odds ratio (e.g., a relative effect of 1.7 indicates a 70% proportional increase in the treatment group relative to that in the placebo group).